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INTRA- AND INTERSPECIFIC DIFFERENCES IN RATE OF OXYGEN CONSUMPTION IN GOBIID FISHES OF THE GENUS GILLICHTHYS [1]

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Many investigators have searched for adaptive physiological differences between intraspecific units. The growing literature has been reviewed a number of times (Thorpe, 1940; Timofeef-Ressovsky, 1940; Dobzhansky, 1951, Ch. 6; Prosser, 1955, 1957; Dehnel, 1955; Moore, 1957).

Recently attention has turned to physiological variation in relation to latitudinal distribution (Roscoff, Station Biologique, 1957; Vernberg 1959a, 1959b; Vernberg and Tashian, 1959). The results from most of the past research of this type cannot be applied to studies of intraspecific divergence (Prosser, 1957). Unfortunately, the early workers either did not consider thermal acclimation (Bullock, 1955, for review), or purposely acclimated the animals to the temperatures at which they were found (*e.g.*, Rao, 1953). Thus latitudinal and acclimation effects have not been clearly separable. Developmental rates of embryos from latitudinally separated populations (Dehnel, 1955) may be independent of latitudinal difference in environment, but only if temperature at the time of yolk formation is without effect on the metabolism of the yolk-consuming embryo. To appreciate genetically adaptive divergence in physiology as a function of latitude, the animals must be acclimated to, and compared at, the same temperatures.

The present study was undertaken to establish the degree of physiological divergence, if any, between widely separated populations of *Gillichthys mirabilis* Cooper. The results of an extensive morphological examination of several populations of this species, and of *Gillichthys seta* (Ginsburg), (Barlow, 1961, and unpublished data) constitute the point of departure for the program at hand. Oxygen consumption, a highly integrated metabolic phenomenon, was the physiological system chosen for the investigation.

[1] A revised portion of the dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of California, Los Angeles. Procedural details, and further figures and tables, are available in the dissertation.

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Material and Methods

Gillichthys seta doubtless is the species most closely related to *G. mirabilis* (Barlow, 1961). In occurrence, *G. seta* is restricted to the northern part of the Gulf of California. It is found only along the exposed coast in the highest intertidal zone among the streams and pools of rocky reefs.

The habitat of *G. mirabilis* is the intertidal region of coastal sloughs. Typically, this is a muddy region exposed to air at low tide. Some of the fish remain at low tide in crab burrows, or the likes, and breathe air; *G. seta* also is capable of aerial respiration.

The distribution of *G. mirabilis* embraces several faunal regions. The northmost populations in central California co-exist with a low-boreal fauna; the southmost populations in the Gulf of California occur within a tropical Panamic region; and the northern part of the Gulf of California, where *G. mirabilis* is also found, has a depauperate, mixed fauna. Thus *G. mirabilis* is arranged in disjunct populations over three roughly parallel, north-south, coast lines.

Distribution along a north-south coast ordinarily corresponds to a temperature gradient, warmer to the south. This applies to the Pacific Coast, and in the winter to the Gulf of California. In the late summer, however, the gradient in the Gulf is either reversed, cooler to the south, or the temperatures are everywhere about the same. As a result, the annual range of mean temperatures in the Gulf of California increases from south to north. The climate of the Salton Sea continues this trend of increased temperature extremes to the north.

In 1930 *G. mirabilis* was introduced into the Salton Sea. The parental stock came from San Diego Bay, just a few miles south of the slough at Los Peñasquitos. The fish have become well established in the Salton Sea in spite of the many peculiar features (Carpelan, 1958) of this environment. Judging from the number of aberrant specimens, the Salton Sea is a marginal habitat for *G. mirabilis*.

Treatment of the animals

The fish were held in sea water in 20- and 40-gallon tanks. The water was aerated, and filtered continuously, and was replaced with fresh sea water two to three days prior to each experiment.

The fish were fed exclusively on fresh squid, which they ate voraciously. The number of days between feedings, as well as the feeding time, were purposely kept irregular to minimize cyclic behavior. They were not fed just prior to an experiment; this interval was adjusted according to the size of the fish and the acclimation temperature (at 10°, intervals of seven to twelve days, and at 24°, of two to four days; longer periods for longer fish), so that the fish when tested were flat-bellied, and production of fecal material was low.

The minimum acclimation time was two weeks at 24° ± 0.5°, and four weeks at 10° ± 0.05°. Experience indicated acclimation was complete sooner than that, especially at 24°. In changing fish from one temperature to the other, they were held for one week at the intermediate temperature of 17°, as was done in the experiments by Brett (1952). Illumination was continuous. For both species, only male or immature fish were used.

Apparatus

Special chambers for the Warburg apparatus were constructed, from ground-glass fittings, to accommodate the elongate fish, and in various sizes (45-cc., 85-cc., and 250-cc.) to maintain the proper ratio between fish and chamber volumes.

Reduction of the oxygen tension in the chamber was circumvented by installing an oxygen well, a collapsible bag made of No. 6, 100 gauge, Saran film, on the air inlet of the manometer. Each time the stopcock was opened to balance the manometer fluid, pure oxygen, at atmospheric pressure, replaced that utilized.

Experimental procedure

Each fish was blotted gently, then weighed in the chamber. Filtered sea water was added in an exact amount, equal to about 5% of the flask volume (enough water to wet the belly and the floor of the mouth of the fish). The flask was then flushed with oxygen, potassium hydroxide solution was quickly introduced into the cup, and the flask was affixed to the manometer. The bath was darkened and the manometers were stationary. Temperatures were controlled to within ±0.025°. The initial temperature was always the same as the acclimation temperature of the fish.

The animals were placed in the bath in the evening, and the first reading was taken twelve hours later between 0830 and 0930 hours. To determine the adjustment period, however, readings were commenced 30 minutes after placing the fish in the bath.

Oxygen uptake was recorded at the acclimation temperature during the thirteenth and fourteenth hours in the chamber. The bath temperature was then steadily raised 7° during one hour. Two hours elapsed at the new temperature before the oxygen uptake was recorded during another two-hour period, the eighteenth and nineteenth hours in the flasks.

Excepting the fish from Puerto San Carlos, no animal was used a second time in the same series of acclimation experiments.

The respiratory rates were compared at 10° and 17° for four populations of *G. mirabilis* acclimated to 10°. From north to south, the fish were from San Francisco Bay (Alviso Ponds), latitude 37° N., in central California; Los Peñasquitos, latitude 33° N., in southern California; the Salton Sea, latitude 33° N.; and San Felipe ("El Marino" slough), latitude 31° N., in the northern part of the Gulf of California. Though the Salton Sea and Los Peñasquitos are on about the same latitude, the warmer climate of the Salton Sea is considered here to be more "southern." San Felipe is not much farther south than southern California but is in a region with a warmer over-all climate.

Fish from the same four places were also acclimated to 24° and tested at 24° and 31°. A group of fish from Puerto San Carlos, latitude 28° N., near Guaymas in the central part of the Gulf of California, was included in the latter comparison. The *G. mirabilis* from San Carlos were small when obtained. In spite of growing well in aquaria, the distribution of sizes of the San Carlos fish included much smaller animals than in the other samples. Consequently they were not included in the analysis of co-variance within the species *G. mirabilis*. Comparisons between *G.*

mirabilis and *G. seta* were based on experiments with *G. mirabilis* from San Carlos, and *G. seta* from Puertecitos, latitude 30° N.

Treatment of the data

The decision to accept or reject the stated statistical hypothesis was based on the 5% critical level in order to minimize type II errors (Hoel, 1954, p. 33). Acceptance of a hypothesis such as equal means does not imply the populations in question were proven to be the same.

The mean rate of oxygen consumption was employed throughout. Minimum rates were only 3% to 5% below the mean.

Regression lines were fitted by least squares to the logarithms of the experimental data. It was convenient to express this rectilinear relationship as oxygen consumption per unit weight, instead of per total weight. The slope is then negative, as indicated in the expression: Rate $= a\ \mathrm{Wt}^{-b}$, where a is the rate when weight (Wt) equals one, and b is the regression coefficient.

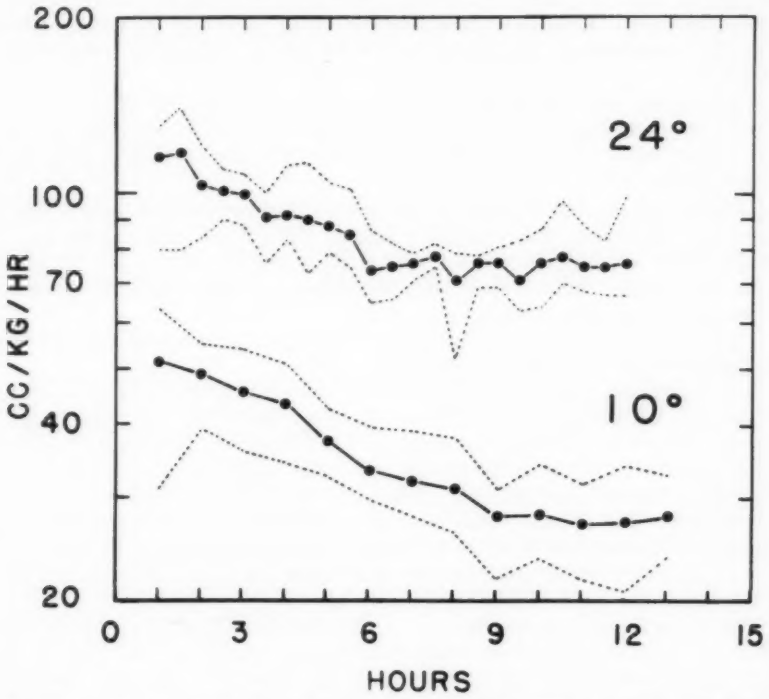

FIGURE 1. Time to steady rate of oxgen consumption for *Gillichthys mirabilis* from Los Peñasquitos. Solid lines connect means and dashed lines connect ranges. Individual rates were adjusted to the eight-gram intercept using regression coefficients of −0.28 for the 10° group and −0.56 for the 24° group (n = 5); the procedure is discussed in Roberts (1957).

Results

Uniformity of results

The rate of oxygen consumption remained the same from month to month in a given population. Fish from the Salton Sea, tested in January, April, and May, showed no perceptible differences. And fish from San Carlos re-tested after periods of growth, continued to manifest rates appropriate to their earlier determined regression lines (Fig. 4).

The oxygen added to the chambers at the beginning of each experiment apparently did not disturb the fish. Controls with air, instead of oxygen, metered into the chambers showed no differences.

At 10° the initial rate of oxygen uptake was high, but reached a constant level within nine to ten hours (Fig. 1). The adjustment time at this temperature was independent of the size of the fish relative to the volume of the chamber.

The initial rate was also high at 24°. For all fish in small or medium size chambers, adjustment was achieved within six to ten hours (Fig. 1). The adjustment time in the largest chambers, however, varied depending on the ratio of the fish size to chamber volume. In the large chambers oxygen consumption was reasonably regular within eight to ten hours if the fish were larger than 15 grams. Because of this, only fish equal to or larger than 20 grams were used in large chambers at 24°.

Wells (1935a) investigated the adjustment time of *G. mirabilis*, but in water instead of air. The results of his four experiments show excellent agreement with

TABLE I

Relation of oxygen consumption to body weight in Gillichthys mirabilis *and* G. seta

Temperature	10° C.			17° C.		
Parameter	n	a	−b	n	a	−b
G. mirabilis						
San Francisco	46	36.9	0.186	34	68.6	0.138
Los Peñasquitos	53	43.7	0.277	43	86.2	0.286
Salton Sea	46	31.0	0.160	36	62.6	0.193
San Felipe	35	57.1	0.346	35	79.5	0.247
Temperature	24° C.			31° C.		
Parameter	n	a	−b	n	a	−b
G. mirabilis						
San Francisco	36	254	0.577	36	300	0.526
Los Peñasquitos	49	256	0.560	40	331	0.530
Salton Sea	36	310	0.626	36	419	0.622
San Felipe	34	246	0.568	34	351	0.613
San Carlos	54	248	0.451	42	318	0.487
G. seta						
Puertecitos	24	177	0.289	24	289	0.257

TABLE II

Results of co-variance tests on differences in rate of oxygen uptake in Gillichthys mirabilis *and* G. seta
Entries refer to G. mirabilis *unless stated otherwise. F = F ratio, d.f. = degrees of freedom,* $_{0.95}$ *= 5% critical value*

	Temperature	F	d.f.	$F_{0.05}$	Decision
Hypothesis: means same after adjustment for body weight					
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	10° C.	8.50	3,175	2.66	Reject
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	17° C.	6.58	3,143	2.67	Reject
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	24° C.	1.93	3,150	2.67	Accept
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	31° C.	5.46	3,141	2.67	Reject
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	24° C.	28.6	1,76	3.97	Reject
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	31° C.	1.07	1,63	3.99	Accept
Hypothesis: Same slope within the groups					
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	10° C.	2.65	3,172	2.66	Accept
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	17° C.	3.58	3,140	2.67	Reject
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	24° C.	0.68	3,147	2.67	Accept
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	31° C.	2.25	3,138	2.67	Accept
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	24° C.	7.65	1,74	3.97	Reject
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	31° C.	12.6	1,62	4.00	Reject

the curves in Figure 1, when recalculated in cc. O_2/kg./hr. and plotted on semi-logarithmic graph paper.

For all groups, the mean rate of oxygen consumption remained consistent over a 12-hour period after adjustment, and no cyclic phenomena were evident. During the readings the rate of uptake of each fish fluctuated, resembling fluctuations noted for other fishes by Spoor (1946) and by Pritchard (1955).

Influence of size on oxygen consumption

As is so often the case (examples in Dehnel, 1960), the smaller the animal, the greater its oxygen consumption per unit weight. In *G. mirabilis* and in *G. seta* this relationship between size and metabolism is more pronounced than in most fishes, the values of *b* being large (see Discussion). Moreover, both inter- and intraspecific differences in the estimate of *b* were found in the genus *Gillichthys*.

The slopes of the lines at 10° were nearly the same for the various populations of *G. mirabilis*, though the differences approached the 5% level of significance (Tables I, II). The slopes for the populations steepened from the northmost (−0.19) to the southmost (−0.35), excluding the Salton Sea group (Table I, Fig. 2), but these differences were of doubtful significance.

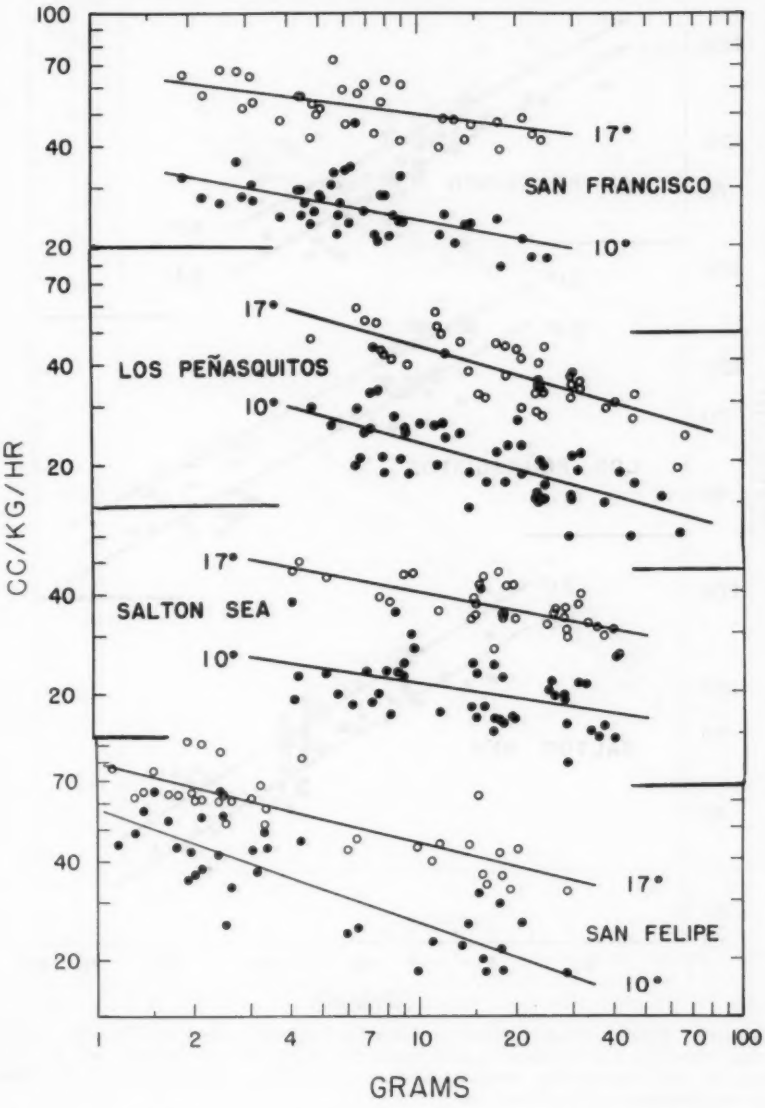

FIGURE 2. Relation between oxygen consumption and body weight of *Gillichthys mirabilis* from San Francisco, Los Peñasquitos, Salton Sea, and San Felipe at 10° (closed circles) and 17° (open circles).

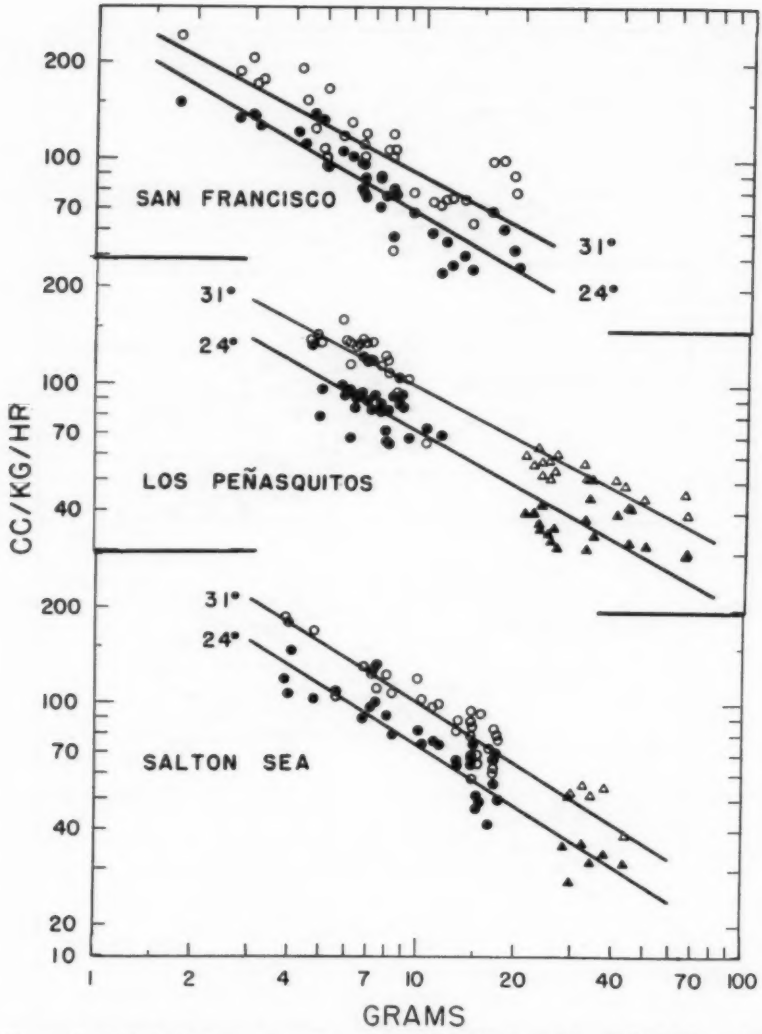

FIGURE 3. Relation between oxygen consumption and body weight of *Gillichthys mirabilis* from San Francisco, Los Peñasquitos, and Salton Sea at 24° (closed symbols) and 31° (open symbols); in small and medium chambers (circles), and in large chambers (triangles). Chamber size not indicated for San Francisco.

At 17° the slopes for the various populations were significantly different (Table II), though the relationship between them was similar to that observed at 10° (Table I, Fig. 2). The value of b was low for fish from San Francisco and Salton

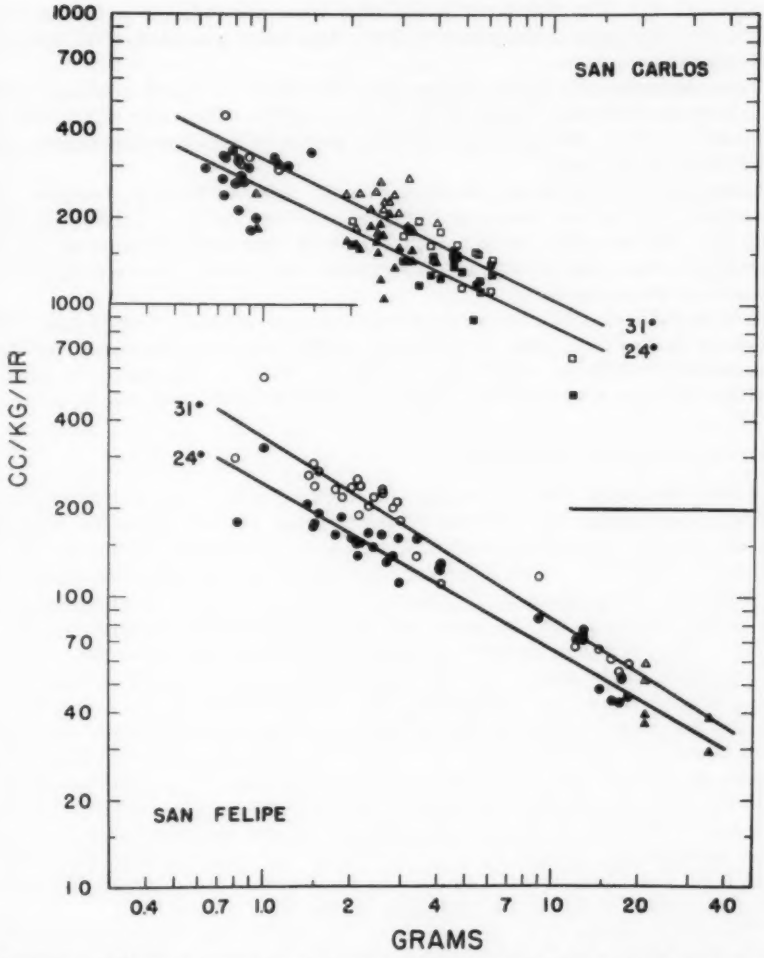

FIGURE 4. Relation between oxygen consumption and body weight of *Gillichthys mirabilis* from San Felipe at 24° (closed symbols) and 31° (open symbols); in small and medium chambers (circles), and in large chambers (triangles); and of *G. mirabilis* from Puerto San Carlos, at 24° (closed symbols) and 31° (open symbols), in small and medium chambers (not indicated); tested on 21 Feb., 1958 (circles), 26 March, 1958 (triangles) and 15 May, 1958 (squares).

Sea, and higher for those from San Felipe and Los Peñasquitos. Thus the increase in temperature sharpened the differences but did not alter their direction. No trend with latitude was noted for the slopes at 17°.

The values of the constant b were statistically uniform at 24° (Tables I, II;

Figs. 3 and 4). The fish from San Carlos were not included in the statistical analysis, but the slope of their data (−0.45) was lower than that of the four principal populations.

Even the stress of a higher temperature, 31°, failed to reveal significant differences between the slopes (Tables I, II; Figs. 3 and 4). The value of *b* for the San Carlos fish (−0.49) diverged from the other groups in the same direction as at 24° in that it was lower again.

Comparisons of the slopes obtained from *G. seta* and from *G. mirabilis* (San Carlos), showed that the two species differed at 24° and at 31° (Tables I, II; Figs. 4 and 5). The separation at 31° was particularly noteworthy because at this temperature the mean rates of oxygen consumption (see below) were not significantly different for the two species.

Both at 24° and at 31°, the constant *b* for young *G. mirabilis* (−0.45 and −0.49) was closer to that of *G. seta* (−0.24 and −0.26) than were the slopes for larger *G. mirabilis* (−0.56 to −0.63, and −0.53 to −0.62). Compared to adult *G. mirabilis*, the slope was lower for smaller *G. mirabilis*, and lower still for *G. seta*.

Mean rates of oxygen consumption

At 10° the mean rates of oxygen consumption of the four populations of *G. mirabilis* were significantly different (Table II, Fig. 2); one regression for all the data also was not statistically acceptable (statistical parameters for this test are not indicated in Table II). This divergence apparently was caused by the smaller fish.

Within the experiments at 17°, the mean rates of oxygen consumption of the four populations were not the same, nor was one regression adequate (Table II, Fig. 2). The divergence, however, seemed to be independent of fish size.

The means of the rates of oxygen consumption at 24° (not including the San Carlos sample) showed no differences, and the hypothesis of one regression line was just acceptable (Table II, Figs. 3 and 4). The larger fish from San Carlos fell on about the same regression as did the fish from the four other populations, whereas the smaller San Carlos fish seemed to lie slightly below this regression.

Under stress of a higher temperature, 31°, differences arose between the mean rates of consumption of the four populations, and one regression also was statistically unsatisfactory (Table II, Figs. 3 and 4). No trend between the means of populations was discernible. The rates of the smaller fish from San Carlos once more appeared to lie below the regression for the other groups.

The oxygen uptake of *G. mirabilis* from San Carlos was compared with that of *G. seta* from Puertecitos. At 24° the means were significantly different, but at 31° the average rates were the same (Table II).

Influence of temperature on oxygen consumption

Four sets of Q_{10} comparisons were made for respiratory rates. Two categories involved the acute changes 10° to 17°, and 24° to 31°. Another category was the comparison of rates at 10° and at 24° after acclimation, the chronic changes. Finally, the rates for fish acclimated to 10° but tested at 17° were contrasted with the rates for fish acclimated to 24° but tested at 31°. These relationships are summarized in Table III. Q_{10} was used only as a descriptive device.

TABLE III

Temperature coefficients (Q_{10}) for Gillichthys mirabilis *and* G. seta

	10° C. to 17° C.		24° C. to 31° C.	
	1 g.	10 g.	1 g.	10 g.
G. mirabilis				
San Francisco	2.42	2.84	1.27	1.50
Los Peñasquitos	2.64	2.56	1.45	1.59
Salton Sea	2.73	2.45	1.45	1.57
San Felipe	1.60	2.22	1.66	1.43
San Carlos	—	—	1.43	1.47
G. seta				
Puertecitos	—	—	2.01	2.24

	10° C. to 24° C.			17° C. to 31° C.		
	1 g.	10 g.	50 g.	1 g.	10 g.	50 g.
G. mirabilis						
San Francisco	3.97	2.09	1.33	2.87	1.52	0.97
Los Peñasquitos	3.53	2.22	1.61	2.62	1.75	1.32
Salton Sea	5.17	2.40	1.41	3.89	1.92	1.17
San Felipe	2.84	1.97	1.52	2.89	1.58	1.04

Rates used in the calculations of the Q_{10} values were obtained from intercepts of the regression lines. Most comparisons were made at the 10-gram intercept because this value was near the median of the size ranges for most samples. The temperature coefficients at the one-gram intercept, too, were listed to facilitate comparisons with other published results. Vagaries of the slope due to chance can be magnified by comparisons made outside the size distribution of the fitted lines, and may lead to erroneous conclusions.

When the temperature was raised from 10° to 17°, the Q_{10} of fish acclimated to 10° had a value greater than 2.0 in all populations at the 10-gram level. This was appreciably higher than the Q_{10}'s recorded for animals acclimated to 24°.

The Q_{10}'s for *G. mirabilis* (10-gram fish) from San Felipe were almost always the lowest for that species. The other Gulf population of *G. mirabilis*, from San Carlos, also had a commensurately low coefficient for the one temperature interval at which it was tested. Thus, the *G. mirabilis* from the Gulf of California had lower temperature coefficients than those from the Pacific Coast, or Salton Sea.

Within the north to south sequence of populations in California (San Francisco, Los Peñasquitos, and Salton Sea), the predominant trend was higher coefficients to the south. But for the acute change in temperature among the 10° acclimation groups, the trend was just the opposite. In general, the values for Los Peñasquitos and Salton Sea were closer to one another than to that for San Francisco, particularly for acute changes.

Gillichthys seta clearly had a higher temperature coefficient than any population of *G. mirabilis* at 24° (Table III). No *G. seta* were tested at 10°.

An additional set of experiments was performed to establish whether or not the

high chronic Q_{10}'s observed in the cold-acclimated fish could be ascribed to the removal of cold depression. The three populations of *G. mirabilis* from California acclimated to 10° were tested at 3.5° intervals from 10° to 27.5°. The program was the same as described in Material and Methods.

Cold depression was not a factor in the temperature response of the cold-acclimated fish. As seen in Figure 6, the Q_{10}'s remained approximately the same between 10° and 24°.

Prosser (1958) has presented a classification of types of metabolic responses to temperature changes in relation to cold- and warm-acclimation. To provide such a comparison, data from a warm-acclimated (24°) group of *G. mirabilis* from San Francisco were included in Figure 6. This curve, however, has been improvised from two different experiments, and requires an explanation.

The points representing rate of oxygen consumption at 24° and 31° were derived from the lines fitted to the results of the standard experiment (Fig. 3), and are reliable.

The values for 17° and 34.5° were obtained as follows. The fish in the respirometers were slowly (eight hours) cooled from 24° to 17°, held at 17° four hours, recorded two hours, then tested at 3.5° intervals up to 34.5°, following the usual program. The values at 17° regularly fell near the projected 24°–31° line. Warming the water to 20.5° and 24°, on the other hand, resulted in pronounced individual differences. These seemed to be of two types: (1) some of the fish manifested high Q_{10}'s, followed by a decrease in rate at a higher temperature, then an increase again at a still higher temperature, whereas (2) the others showed what could be called the anticipated regular increment. I have exercised my judgment and selected out those data that I believe to be reliable, *i.e.*, all the recordings from the San Francisco fish taken at 17° and 34.5°, but none in between.

The change in Q_{10} revealed by a comparison of the two kinds of curves in Figure

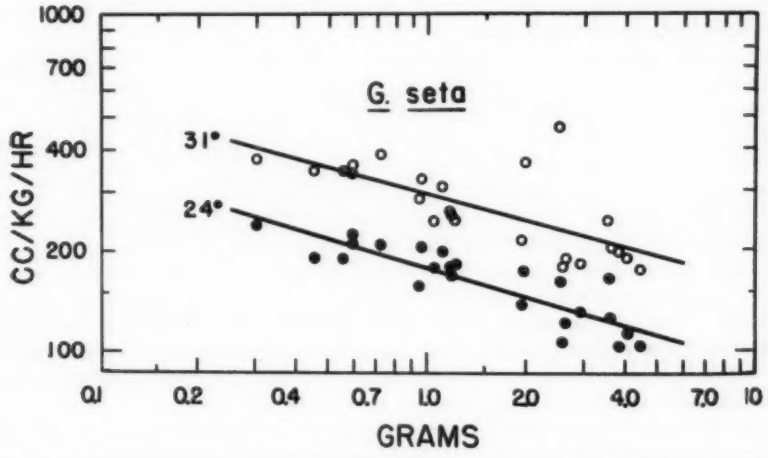

FIGURE 5. Relation between oxygen consumption and body weight of *Gillichthys seta* from Puertecitos at 24° (closed circles) and 31° (open circles).

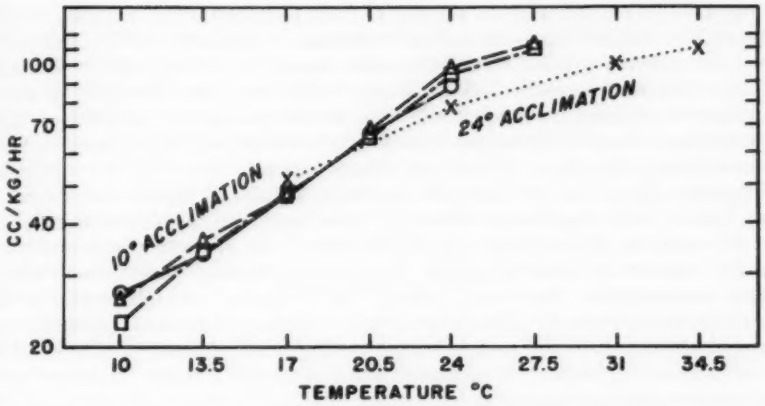

FIGURE 6. Influence of cold- and warm-acclimation on metabolic responses of *Gillichthys mirabilis* to temperature changes. Each point represents the mean rate of oxygen consumption for ten fish, weighing from 4 to 10 grams. Individual rates were adjusted to the 8-gram intercept using the appropriate values of *b* listed under 10° and 24° in Table I; the procedure is discussed in Roberts (1957). Acclimation to 10°: circle = San Francisco population, triangle = Los Peñasquitos population, square = Salton Sea population. Acclimation to 24°: X = San Francisco population.

6 would be classified in the system proposed by Prosser (1958) as Type III B, rotation counter-clockwise with cold acclimation. Clockwise rotation evidently is more common than counter-clockwise (Prosser, 1958). The curves for warm- and cold-acclimated fish intersect between 17° and 20.5°. The curve (not shown) for the warm-acclimated fish from Los Peñasquitos intersects the curves for cold-acclimated animals at 20.5°.

Evidently little or no translation is involved. This would be more apparent if the warm-acclimated fish had been tested over a lower temperature range. Incidentally, above 34° the oxygen uptake in many of the 24°-acclimated fish decreases, and between 35° and 37° many die (further experiments not reported here).

Influence of size on temperature coefficients

There was no apparent difference in Q_{10} between large and small *G. mirabilis* for acute temperature changes. But the temperature coefficients after acclimation were influenced greatly by the size of the fish involved. The chronic Q_{10}'s were strikingly higher in the smaller fish (Table III; Figs. 2 to 4). Apparently acclimation was more effective in larger fish. The 50-gram fish, acclimated to 24° and tested at 31°, commonly had lower respiratory rates than fish of the same size acclimated to 10° and tested at 17°. The size of the fish, therefore, is important in chronic, but not acute, metabolic adjustments.

DISCUSSION

Evidence in fishes for intraspecific differentiation of adaptive physiological systems has received infrequent but notable attention. Heuts (1947, 1956) described

the physiological adaptations to salinity and temperature in the embryos of three races of the stickleback, *Gasterosteus aculeatus*. Minamori (1957, and earlier papers cited there) studied in detail various aspects of developmental physiology in races of the spinous loach, *Cobitus taenia*. The races were arranged in a series according to temperature adaptation. The forms ecologically adapted to higher temperatures, tolerated higher temperatures and developed more slowly at intermediate temperatures than did the cold-adapted forms. Hart (1952) investigated geographic variation in heat tolerance in twelve species of fresh-water fishes. In three species, only, significant differences were apparent between subspecies. In field experiments, Wohlschlag (1957) discovered the sympatric, but ecologically separate forms of an arctic whitefish, *Coregonus sardinella*, had different rates of oxygen consumption. But these "forms" of *Coregonus* probably would be considered distinct species by Svärdson (1953). Guppies, *Lebistes reticulatus*, from inbred lines had different susceptibilities to high temperatures (Gibson, 1954). Under extreme environmental conditions some genotypes of carp, *Cyprinus* (sp.?), were more viable than others (Kirpichnikov, 1945).

Differences between populations, after acclimation, usually are attributed to genetic dissimilarities. The evidence for this thesis is indirect, but consistent (references in Introduction, and Fry, 1957a). The role of the environment in modifying the degree of reversibility, however, is not known.

The temperatures associated with early development allegedly can alter the thermal resistance of guppies, *Lebistes reticulatus* (Gibson, 1954). The thermal resistance of fish reared at 25° and at 30° was similar, but those cultured at 20° were less resistant. At the lowest temperature only two female guppies could be induced to give birth, and at the highest temperature mortality was appreciable. The fish reared at the high, and especially at the low, temperatures could have been selected for by the effects of the thermal environment. Other experiments in the same paper demonstrated different heat resistances in different genetic lines. Males and females also had dissimilar tolerances, and the sex ratios in the experiments on thermal history were not given. The influence of the early thermal experiences on the metabolism of adult poikilotherms remains, then, an open question.

The results reported here, nonetheless, are interpreted as though metabolic differences between fully acclimated animals from naturally occurring populations, indicate heritable dissimilarities.

Influence of size on oxygen consumption

Although the regression coefficients relating oxygen consumption to weight were not especially informative at the intraspecific level, they were most revealing for species relationships. The slope of the regression for the small species, *G. seta*, obviously was lower than that for the adults of *G. mirabilis* (24° acclimation). The disparity was less apparent when the data from adult *G. seta* were compared with those for sub-adult *G. mirabilis*. As noted below, the value of b (*i.e.*, $-b$) tended to decrease at small sizes in *G. mirabilis* (acclimated to 24° only). Morphologically, adult *G. seta* retain many characteristics which in *G. mirabilis* are juvenile (Barlow, 1961). The low regression coefficients for oxygen uptake in *G. seta* might also represent a retention of a juvenile feature.

Von Bertalanffy (1951) discussed the relation between regression coefficients

and phylogeny. He opined that some higher taxonomic categories could be characterized by the value of *b*. Apparently this does not hold for gobiid fishes since the slopes differ between species, and change with acclimation as well.

Certain difficulties were encountered with the data obtained from fish acclimated to 24°. The relationship between the logarithms of oxygen consumption and body weight was not strictly rectilinear over the size range of the experimental animals. There was a tendency for the negative value of *b* to decrease slightly in the smaller *G. mirabilis*, and also in the large adults, though less so. As Zeuthen (1955) suggested, it may be best to regard *b* as a tangent to some more complicated curve.

To complicate matters, the value of *b* in *G. mirabilis* acclimated to 10° was relatively constant over the entire size range of the experimental animals. The low temperature of 10° is a normal winter temperature for *G. mirabilis*, but surely it is suboptimal for this species in terms of activity and growth; the fish seem well suited to 24°. Possibly, the low temperature of 10° effects a physiological compensation for the unnatural continuous illumination. Or, at 10° metabolism is less affected by growth and activity. The values of *b* obtained from the cold-acclimated fish are more in accord with most of those reported for other fishes.

Zeuthen (1953) suggested that −0.22 probably is a representative value for *b* among fishes. Fry (1957b) cited −0.10 to −0.20 as usual values for *b*, but also noted five instances where values of from −0.33 to −0.50 had been reported. The value of *b*, −0.50 to −0.60 for *G. mirabilis* acclimated to 24°, then, is higher than that usually considered typical for fishes. The value of *b* (−0.15) reported by Scholander *et al.* (1953), was derived from a composite of several species. The data for some of the species, however, appear to show a slope greater than −0.15 (*Abudefduf saxatilus, Scarus croicensis*). Wohlschlag (1957) recorded a slope of −0.60 for a coregonid fish, though he attributed the high value to the small size of the sample. Recent investigations into other animal groups have revealed values of *b* well above −0.33 predicted from the surface volume relationship (references in Zeuthen, 1953; Mann, 1956; Dawson and Bartholomew, 1956; Vernberg, 1959a; Dehnel, 1960; Velma Vance, personal communication).

Mean rates of oxygen consumption

Physiological responses to stress may reveal differences when measurements within the temperature range to which the animals are adjusted might fail (Prosser, 1957). The means of the rates of oxygen consumption in *G. mirabilis* were the same only at 24°, which probably lies within its optimal temperature range. Differences were apparent when the temperature was raised to 31°. The lowest temperature, 10°, must have been stressful: activity was greatly reduced, although social behavior, frontal display by males, was observed. Fish acclimated to 17° were only slightly less active than those at 24°. Nonetheless, the sudden 7° increase in temperature from 10° to 17° probably stressed the metabolic system of the cold-acclimated fish.

While differences in mean rates of oxygen consumption at a given temperature appeared to indicate divergences between populations of *G. mirabilis*, the direction of the departures seemed to be irregular. At 10°, only, there may have been evidence for higher metabolic rates to the south in smaller fish. This was particularly noticeable in the animals from San Felipe, that had been tested in late spring. The

other populations had been examined during the winter. But if there had been a residual seasonal adjustment, the rates for the San Felipe group should have been low in comparison to the winter groups. As seen, just the opposite occurred.

The oxygen consumption of *G. mirabilis* is similar whether the fish are in or out of water. Though not measured in water, the rates reported here for aerial respiration of *G. mirabilis* agree with those found by Wells (1935a) for respiration in water of the same species, when allowances are made for differences in thermal acclimation. The rate of oxygen uptake of the Hungarian mud-minnow, *Umbra lacustris* (*krameri?*), also is the same whether entirely aquatic, or a combination of aquatic and aerial (Geyer and Mann, 1939).

The rates of oxygen uptake in *Gillichthys mirabilis* and *G. seta* accord with the values reported for other species of air-breathing Gobiidae. The rates at 24.5° for *Periophthalmus vulgaris* (seven grams) and *P. dipus* (16 and 17 grams) given by Schöttle (1931) coincide nicely with the regression relating oxygen consumption to weight for *G. mirabilis*. The rate for *P. schlosseri* (110 grams) was about 100% higher than would have been expected for *G. mirabilis* of that size, but agreed well with the extension of the regression for *G. seta*. The data of Schöttle were reduced by 50% before making the comparisons because she recorded the rates during the first hour; the values for *G. mirabilis* decreased by about this amount during the 12-hour adjustment period.

Influence of temperature on oxygen consumption

The responses to temperature changes seemed to be of more adaptive significance than the absolute level of oxygen consumption. While the mean rates showed no interpretable differences, the disposition of the Q_{10}'s suggested a meaningful pattern.

The temperature coefficient data infer the following arrangement of populations: *G. mirabilis* from the north and central regions of the Gulf of California are alike, but differ from the other groups considered by having consistently lower Q_{10}'s; *G. mirabilis* from San Francisco Bay are similar to, but somewhat apart from, the other two populations in California in that their Q_{10} is highest at low temperatures, but relatively low at high temperatures; *G. mirabilis* from the Salton Sea and Los Peñasquitos show a correspondence when responses to acute changes are compared, but are dissimilar when temperature coefficients of acclimated rates are considered.

Temperature coefficients generally are higher with adaptation to higher temperatures (but see Dehnel, 1960): northern species or forms usually have lower coefficients than their southern counterparts (Rao and Bullock, 1954; Minamori, 1957; Heuts, 1956; Tashian, 1956; Dehnel, 1955). In some respects *G. mirabilis* was just the opposite: warm-acclimated fish had low Q_{10}'s in all the groups, and cold-adapted ones had high Q_{10}'s. A further inconsistency was that the Q_{10}'s were higher in northern populations when the fish were cold-acclimated. Certain data, however, were in accord with the hypothesis of higher Q_{10}'s in more southern populations. Warm-acclimated *G. mirabilis* from the Pacific Coast and Salton Sea had higher Q_{10}'s to the south. But the two Gulf of California populations had even lower coefficients than the northmost Pacific Coast population, San Francisco. Certainly the Gulf habitats experience much higher maximum temperatures and a greater range, too, than do those in San Francisco Bay. The temperature patterns in the Gulf and on the Pacific Coast are not equatable.

The most obvious assumption is that the low sensitivity to temperature changes observed in *G. mirabilis* from the Gulf of California is an adaptation to the more variable climate there. Yet *G. seta* from the upper Gulf lives in a habitat subject to even greater thermal oscillations; and the Q_{10}'s for *G. seta* are higher than for any population of *G. mirabilis* under the same conditions. This may not be a fair comparison because it involves another species with different behavior and ecology.

The adaptive significance of the nature of the Q_{10}'s in *G. mirabilis,* and in *G. seta,* cannot be appreciated until more information is at hand, both with regard to the general and the specific aspects of the problem. It might be presumed, though, that within the species *G. mirabilis* low sensitivity to temperature change is an adaptation to higher temperatures, and high sensitivity an adjustment to lower temperatures. This is at variance with the conclusions of Scholander *et al.* (1953) which were based on interspecific comparisons, and also with the hypothesis put forth by Rao and Bullock (1954).

Influence of size on temperature coefficients

The few available data for fishes indicate that size affects Q_{10} in different ways depending on the particular species, and probably on the experimental procedures. The temperature coefficient was said to be independent of size in goldfish, *Carassius auratus* (Fry and Hart, 1948). Within a given race of the loach, *Cobitis taenia,* temperature coefficients of embryos were independent of egg volume (Minamori, 1957). At low temperatures size had no role in the determination of Q_{10}'s in the killifish, *Fundulus parvipinnis,* but the smaller fish were more sensitive at higher temperature intervals (Wells, 1935b; re-evaluated on the basis of relative changes). In another cyprinodontid fish, *Crenichthys baileyi,* from a warm spring, small individuals were more sensitive to a temperature drop than were large ones (Sumner and Lanham, 1942). In the brook trout, *Salvelinus fontinalis,* size had little influence on the response to chronic changes in temperature when standard metabolic rates were compared, except at the highest temperature interval where small fish were more responsive. When active, however, the smaller trout had higher temperature coefficients than the larger ones (Job, 1955). Measured by opercular rates, large carp, *Cyprinus carpio,* were more responsive to long term (chronic) temperature changes than were small individuals (Meuwis and Heuts, 1957).

In *Gillichthys mirabilis,* and in *G. seta,* temperature coefficients were not size-dependent in short term (acute) temperature changes. The relatively greater increase in respiratory rate of small *G. mirabilis* acclimated to 24° as contrasted to 10° may indicate that large *G. mirabilis* are metabolically more homeostatic than small, though only when allowed time to adjust. Meuwis and Heuts (1957) noticed a similar relationship in the carp, except the smaller fish, not the larger, were more homeostatic.

Comparison of physiological and morphological findings

The two species involved, *G. mirabilis* and *G. seta,* are distinct but difficult to distinguish from one another (Barlow, 1961). Ichthyologists, unless experienced with this group, frequently have difficulties separating them. In many respects *G. seta* represents an extension of the morphological cline manifested by the widely

distributed populations of *G. mirabilis* (Barlow, unpublished data). Physiologically, however, the two species diverge where their distribution overlaps, the Q_{10}'s and the regression coefficients, *b*, showing marked character displacement.

Within the species *G. mirabilis*, the greatest structural differences between populations were for the most part discernible only by means of statistical analysis. The aberrant Salton Sea population, of course, was an exception, but need not disturb the thesis being put forward here. Furthermore, the morphological investigation involved the analysis of many characters, such as measurements of several body parts, counts of the various fins and the vertebrae, and extent and nature of squamation. Among the multitude of possible physiological parameters, only oxygen consumption was studied. Considering this, it is fair to say the magnitude of intraspecific variation in oxygen uptake was commensurate with that observed in the morphological study.

The pattern of divergence, the "species structure" of *G. mirabilis*, became apparent only when many intervening populations, in this case 17, were compared morphologically. It is questionable if much sense could have been made of the morphological data from only those five populations analyzed experimentally. Indeed, before the series of populations had been investigated, three nominal species had been proposed for the two under discussion, and the situation was not at all clear (Barlow, 1961).

Beyond this, it might be pertinent to ask if the experiments were designed properly to reveal meaningful differences in the respiratory rates. Perhaps more informative data could be obtained by acclimating the animals to a controlled regime of oscillating temperatures, simulating the natural situation. After all, the most obvious thermal dissimilarity in the locales involved is the range of temperatures experienced by each, not the mean. Constant temperature, not to mention illumination, is an unreal circumstance for the animals; the absence of these modulating influences might even be inimical to certain metabolic processes.

In closing, the lack of differences in the nature of the oxygen uptake at 24° should be emphasized. From numerous field observations, I believe this temperature is near the thermal optimum for the species *G. mirabilis* throughout its geographic range. The similarities at 24°, therefore, might have been expected. The dissimilarities in the populations were manifested as the temperature departed from this value. The environment of each population evidently is unique in its combination of various temperatures (especially maxima and minima), photoperiod, salinity, and so on. Probably the singular features of each habitat have stamped their character on the gene pool of the population, effecting a slight, though significantly adaptive, divergence in the metabolic response to temperature.

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Summary

1. Rate of oxygen uptake was determined for moist fish in a Warburg apparatus. Fish acclimated to 10° were tested at 10° and 17°; those acclimated to 24° were tested at 24° and 31°. Five populations of *G. mirabilis* were investigated (San Francisco Bay in central California, Los Peñasquitos in southern California, San Felipe in the northern Gulf of California, Puerto San Carlos in the central Gulf, and the Salton Sea). One population of *G. seta* (from the upper Gulf) was studied at 24°.

2. Rate of oxygen consumption per unit weight was plotted against body weight. The regression coefficient, b, differed between populations for *G. mirabilis* though not in an interpretable fashion. The value of b was low at 10° and 17° (−0.14 and −0.35), and high at 24° and 31° (−0.45 and −0.63). The slopes were much lower for *G. seta* at 24° and 31° (−0.29 and −0.26) than for *G. mirabilis*.

3. The mean rates of oxygen uptake were different for the populations of *G. mirabilis* at all temperatures except 24°. The average rate for *G. seta* at 24° was lower than that for *G. mirabilis*, but the rates were about the same for both species at 31°.

4. The temperature coefficients, Q_{10}, varied from population to population of *G. mirabilis* in what seemed to be a pattern. The coefficients were lower in groups from the Gulf of California at almost all temperature intervals. Within the Pacific Coast and Salton Sea populations, the Q_{10}'s for the northern population were highest at the low acclimation temperature, and lowest at the high acclimation temperature. For all groups, the Q_{10}'s were highest when cold-acclimated (2.2 to 2.8), and lowest when warm-acclimated (1.4 to 1.6). The coefficient for warm-acclimated *G. seta* was high (2.0).

5. There was no apparent difference in temperature coefficients between large and small *G. mirabilis* for acute temperature changes. The smaller fish manifested appreciably higher Q_{10}'s than did the larger fish, however, when rates were compared after acclimation.

6. The degree of intra- and interspecific differences in the nature of the oxygen consumption was roughly of the same order of magnitude as the morphological differences reported in another article.

Literature Cited

Barlow, G. W., 1961. Gobies of the genus *Gillichthys*, with a comment on the sensory canals as a taxonomic tool. *Copeia*, in press.

von Bertalanffy, L., 1951. Metabolic types and growth types. *Amer. Nat.*, **85**: 111–117.

Brett, J. R., 1952. Temperature tolerance in young Pacific salmon, genus *Oncorhynchus*. *J. Fish. Res. Bd. Can.*, **9**: 265–323.

Bullock, T. H., 1955. Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.*, **30**: 311–342.

Carpelan, L. H., 1958. The Salton Sea. Physical and chemical characteristics. *Limnol. Oceanog.*, **3**: 373–386.

Dawson, W. R., and G. A. Bartholomew, 1956. Relation of oxygen consumption to body weight, temperature, and temperature acclimation in lizards *Uta stansburiana* and *Sceloporus occidentalis*. *Physiol. Zoöl.*, **29**: 40–51.

Dehnel, P. A., 1955. Rates of growth of gastropods as a function of latitude. *Physiol. Zoöl.*, **28**: 115–144.

DEHNEL, P. A., 1960. Effect of temperature and salinity on the oxygen consumption of two intertidal crabs. *Biol. Bull.*, **118**: 215–249.

DOBZHANSKY, T. G., 1951. Genetics and the Origin of the Species. Columbia University Press, New York, 364 pp.

FRY, F. E. J., 1957a. The lethal temperature as a tool in taxonomy. *L'Ann. Biol., 3rd ser.*, **33**: 205–219.

FRY, F. E. J., 1957b. The aquatic respiration of fish. *In:* The Physiology of Fishes, M. E. Brown, ed., Acad. Press, New York, Vol. 1, pp. 1–63.

FRY, F. E. J., AND J. S. HART, 1948. The relation of temperature to oxygen consumption in the goldfish. *Biol. Bull.*, **94**: 66–77.

GEYER, F., AND H. MANN, 1939. Beiträge zur Atmung der Fische II. Weiteres zur Atmung des ungarischen Hundsfisches (*Umbra lacustris* Grossinger). *Zool. Anz.*, **127**: 305–312.

GIBSON, M. B., 1954. Upper lethal temperature relation of the guppy, *Lebistes reticulatus*. *Canad. J. Zool.*, **32**: 393–407.

HART, J. S., 1952. Geographic variations of some physiological and morphological characters in certain freshwater fishes. *Publ. Ontario Fish. Res. Lab.*, (**72**): 1–79.

HEUTS, M. J., 1947. Experimental studies on adaptive evolution in *Gasterosteus aculeatus* L. *Evolution*, **1**: 89–102.

HEUTS, M. J., 1956. Temperature adaptation in *Gasterosteus aculeatus* L. *Pubbl. Sta. Zool. Napoli*, **28**: 44–61.

HOEL, P. G., 1954. Introduction to Mathematical Statistics. Wiley and Sons, New York, 331 pp., 2nd ed.

JOB, S. V., 1955. The oxygen consumption of *Salvelinus fontinalis*. *Toronto Univ. Biol. Ser.*, (61); *Publ. Ontario Fish. Res. Lab.*, (**73**): 1–39.

KIRPICHNIKOV, V. S., 1945. Viability, rate of growth and morphology of carps of different genotypes as affected by rearing conditions. *C. R. Acad. Sci. U. R. S. S.*, **47**: 503–506.

MANN, K. H., 1956. A study of the oxygen consumption of five species of leech. *J. Exp. Biol.*, **33**: 615–626.

MEUWIS, A. L., AND M. J. HEUTS, 1957. Temperature dependence of breathing rate in carp. *Biol. Bull.*, **112**: 97–107.

MINAMORI, S., 1957. Physiological isolation in Cobitidae. VI. Temperature adaptation and hybrid inviability. *J. Sci. Hiroshima Univ., Ser. B., Div. 1*, **17**: 66–119.

MOORE, J. A., 1957. An embryologist's view of the species. *In:* The species problem, E. Mayer, ed., *Publ. Amer. Ass. Advanc. Sci.*, (**50**): 325–338.

PRITCHARD, A., 1955. Oxygen requirements of some Hawaiian tuna baitfish. *U. S. Fish and Wildl. Serv., Spec. Sci. Rept., Fish* (**145**): 1–30.

PROSSER, C. L., 1955. Physiological variation in animals. *Biol. Rev.*, **30**: 229–262.

PROSSER, C. L., 1957. The species problem from the viewpoint of a physiologist. *In:* The species problem, E. Mayer, ed., *Publ. Amer. Ass. Advanc. Sci.*, (**50**): 339–369.

PROSSER, C. L., 1958. General summary: The nature of physiological adaptation. *In:* Physiological Adaptation, C. L. Prosser, ed., American Physiological Society, Washington, D. C., pp. 167–180.

RAO, K. P., 1953. Rate of water propulsion in *Mytilus californianus* as a function of latitude. *Biol. Bull.*, **104**: 171–181.

RAO, K. P., AND T. H. BULLOCK, 1954. Q_{10} as a function of size and habitat temperature in poikilotherms. *Amer. Nat.*, **88**: 33–44.

ROBERTS, J. L., 1957. Thermal acclimation of metabolism in the crab *Pachygrapsus crassipes* Randall. II. The influence of body size, starvation, and molting. *Physiol. Zoöl.*, **30**: 232–242.

ROSCOFF, STATION BIOLOGIQUE, 1957. Colloque International de Biologie Marine. *L'Ann. Biol., 3rd ser.*, **33**.

SCHOLANDER, P. F., W. FLAGG, V. WALTERS AND L. IRVING, 1953. Climatic adaptation in arctic and tropical poikilotherms. *Physiol. Zoöl.*, **26**: 67–92.

SCHÖTTLE, E., 1931. Morphologie und Physiologie der Atmung bei wasser-, schlamm- und landlebenden Gobiiformes. *Zeitschr. wiss. Zool.*, **140**: 1–115.

SPOOR, W. A., 1946. A quantitative study of the relationship between the activity and oxygen consumption of the goldfish, and its application to the measurement of respiratory metabolism in fishes. *Biol. Bull.*, **91**: 312–325.

SUMNER, F. B., AND U. N. LANHAM, 1942. Studies on the respiratory metabolism of warm and cool spring fishes. *Biol. Bull.*, **82**: 313–327.

SVÄRDSON, G., 1953. The coregonid problem. V. Sympatric whitefish species of the lakes Idsjön, Storsjön, and Hornayan. *Inst. Fresh-water Res. Drottningholm*, (**34**): 141–166.

TASHIAN, R. E., 1956. Geographic variation in the respiratory metabolism and temperature coefficient in tropical and temperate forms of the fiddler crab, *Uca pugnax*. *Zoologica*, **41**: 39–47.

THORPE, W. H., 1940. Ecology and the future of systematics. *In:* The New Systematics, J. S. Huxley, ed., Clarendon Press, Oxford, pp. 341–364.

TIMOFEEFF-RESSOVSKY, N. W., 1940. Mutations and geographical variation. *In:* The New Systematics, J. S. Huxley, ed., Clarendon Press, Oxford, pp. 73–136.

VERNBERG, F. J., 1959a. Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca*. II. Oxygen consumption of whole organisms. *Biol. Bull.*, **117**: 163–184.

VERNBERG, F. J., 1959b. Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca*. III. The influence of temperature acclimation on oxygen consumption of whole organisms. *Biol. Bull.*, **117**: 582–593.

VERNBERG, F. J., AND R. E. TASHIAN, 1959. Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca*. I. Thermal death limits. *Ecology*, **40**: 589–593.

WELLS, N. A., 1935a. Change in rate of respiratory metabolism in a teleost fish, induced by acclimatization to high and low temperature. *Biol. Bull.*, **69**: 361–367.

WELLS, N. A., 1935b. The influence of temperature upon the respiratory metabolism of the Pacific killifish, *Fundulus parvipinnis*. *Physiol. Zoöl.*, **8**: 196–227.

WOHLSCHLAG, D. E., 1957. Differences in metabolic rates of migratory and resident freshwater forms of an arctic whitefish. *Ecology*, **38**: 502–510.

ZEUTHEN, E., 1953. Oxygen uptake as related to body size in organisms. *Quart. Rev. Biol.*, **28**: 1–12.

ZEUTHEN, E., 1955. Comparative physiology (respiration). *Ann. Rev. Physiol.* **17**: 459–482.

THE ABSENCE OF DAILY PHOTOSYNTHETIC RHYTHM IN SOME LITTORAL MARINE ALGAE

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A photosynthetic periodicity in algae has been reported by several workers in recent years. Investigations with natural populations of planktonic forms have shown a marked variation in the ability of these organisms to fix carbon dioxide at different times of day. The rate of photosynthesis is reportedly highest in the middle and late morning, and lowest in the late afternoon and evening, a difference of several fold occurring (Doty and Oguri, 1957; Yentsch and Ryther, 1957; Shimada, 1958). In the coenocytic alga *Hydrodictyon* it has been found that a photosynthetic rhythm established with light-dark cycles persists for two or three periods in continuous light, the period of the induced rhythm depending upon that of the previous light-dark regime; thus, it is in this case not an inherent but an imposed or conditioned rhythm (Pirson, 1957). In *Chlorella* it has been found possible to establish synchronous cultures by means of light-dark cycles (Tamiya, 1957; Sorokin, 1960). In this case the rate of photosynthesis varies with the age of the cells, younger cells in general showing a rate higher than that of older cells. In view of these observations it was thought desirable to determine whether some of the multicellular algae show a photosynthetic rhythm; therefore, the following experiments were carried out at the Hopkins Marine Station during June and July of 1960.

Materials and Methods

The algae utilized in this study were common littoral marine genera collected in the vicinity of Pacific Grove, California. The organisms were maintained in running sea water in a laboratory for a period of not greater than 24 hours prior to the start of the tests. The tissues were placed in one-liter jars completely filled with natural sea water and exposed for a period of one-half hour to incandescent light (2 bulbs) of an intensity approximating 800 foot candles. The temperature within the sampling jars was held constant at about 15° C. by submerging them in a battery jar through which sea water was circulated rapidly during the course of the light exposure. The sampling jars were turned around once during the exposure period to stir the contents. At the conclusion of the exposure, the oxygen content of the water within the sampling jars was determined by the Winkler technique, and the rate of oxygen production per gram fresh weight calculated. Values presented are for net oxygen production, uncorrected for respiration; however, since respiration is low in relation to photosynthesis at the light intensity used (about 10% as shown by occasional dark-bottle checks), variations in respiration would be small

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compared to total oxygen production. In these experiments the initial exposure was made in the middle of the morning between 9:00 and 10:30 A.M., the second exposure in the early afternoon between 12:00 and 1:30 P.M., and the third in the late afternoon between 4:30 and 6:30 P.M. The final exposure was made the next morning between 6:30 and 9:00 A.M., the same piece of tissue being used in all four exposure periods.

Two of the genera, *Ulva* and *Porphyra*, were subjected to cycles of 12 hours of white light followed by 12 hours dark for a period of four to ten days to see if a photosynthetic rhythm could be induced in the same manner as in *Hydrodictyon* and *Chlorella*. After the light-dark treatment, the rate of photosynthesis with continuous light and with intermittent light (5 minutes light, 5 minutes dark) was measured by affixing the tissue to a platinum electrode as described previously (Haxo and Blinks, 1950). An electrical potential (0.5 volt) was applied to the electrode, the rate of oxygen production being reflected in the current flow. The latter was recorded on a Varian Graphic Recorder. A flow of sea water around the tissue was maintained throughout this experiment. The light intensity was approximately 80 foot candles.

Results

The results of experiments with four of the genera tested for a naturally occurring diurnal rhythm are summarized in the accompanying table. It is clear that no rhythm such as that observed in the plankton was detected in the organisms tested here. In addition to the species listed in the table, the following organisms were tested and did not show any photosynthetic rhythm: *Prionitis lanceolata, Endocladia muricata, Gigartina papillata, G. canaliculata, Porphyra perforata, Rhodoglossum affine, Fucus furcatus, Coilodesme californica,* and *Egregia menziesii*. In some cases small variations of 5 to 10% were observed, but these are minor compared to the differences of several fold reported for the plankton and for *Hydrodictyon*. In a few cases the late afternoon value was significantly lower than that of the previous morning, but in these cases an even lower value was observed the next morning, indicating poor survival of the tissue rather than a rhythm.

The samples of *Porphyra* and *Ulva* subjected to the regular light-dark cycles also showed no subsequent photosynthetic rhythm. In the case of *Porphyra* the ability to photosynthesize in either intermittent or continuous light did not change during the course of the day. In *Ulva* the rate of photosynthesis was at first high on exposure to light after a dark period of several hours, gradually dropping to a lower steady level after about two hours of constant or intermittent illumination. The lower level was maintained as long as the illumination was continued, so that

Table I

Rate of oxygen production in cubic centimeters per hour per gram fresh weight

Organism	Morning	Early afternoon	Late afternoon	Next morning
Hesperophycus harveyanus	.15	.19	.19	.19
Chaetomorpha aerea	.35	.30	.38	.33
Ulva sp.	1.08	1.16	1.10	1.16
Iridaea flaccida	.34	.38	.35	.35

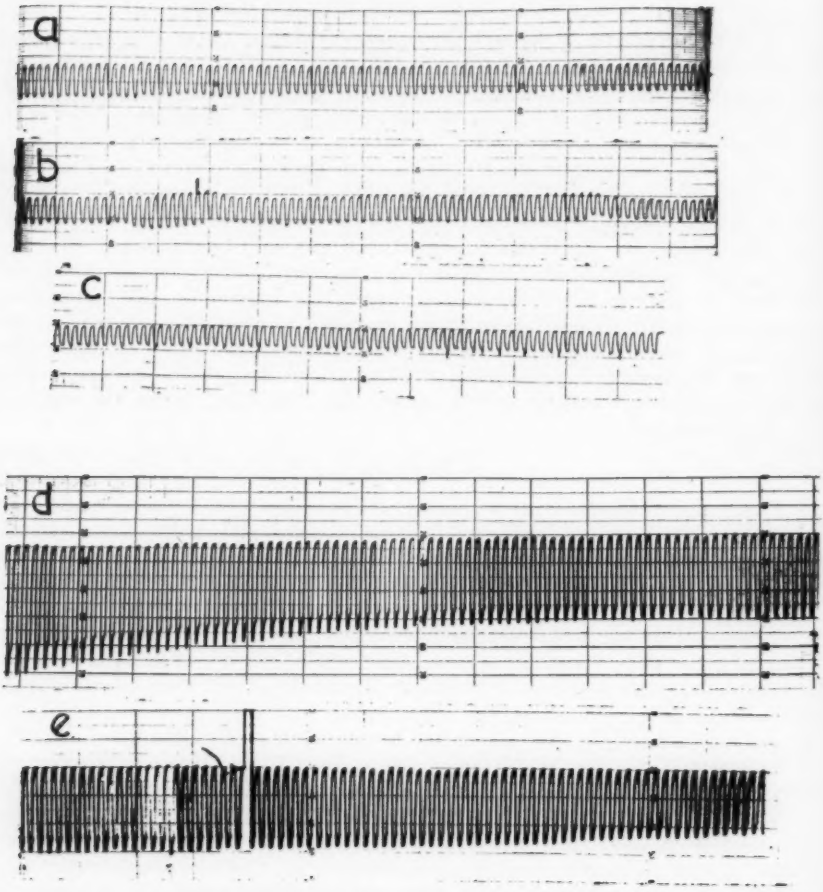

FIGURE 1. Recording of photosynthetic oxygen production by *Ulva* sp. in intermittent light (80 F.C.); 5 minutes light alternated with 5 minutes dark. a, b, and c constitute a continuous 40-hour sequence; d and e another 28-hour sequence. No diurnal rhythm is apparent, only a slow falling away of the original photosynthetic rate. Tissues were previously exposed to 12 hours light, 12 hours dark for 10 days. Time marks one hour apart; downward excursion of the recorder is proportional to oxygen production.

no rhythm was occurring. The accompanying figure shows typical patterns displayed by *Ulva*. Similar records were taken for *Porphyra*.

DISCUSSION

It is apparent from the results presented here that no diurnal rhythm occurs in the organisms tested, and that in *Ulva* and *Porphyra* no photosynthetic periodicity is

induced by a 12 hour light-12 hour dark regime. In the latter connection, no information is available as to whether or not cell division is synchronized by the light-dark cycles, although the absence of any detectable photosynthetic rhythm tends to argue against such synchronization. No explanation appears to be at hand for the diurnal periodicity reported by Doty and Oguri and others. The rhythm in *Hydrodictyon* may be the result of physiological differences in the cells at different stages of development, as Tamiya (1957) has suggested.

LITERATURE CITED

Doty, M. S., and M. Oguri, 1957. Evidence for a photosynthetic daily periodicity. *Limnol. Oceanogr.*, **2**: 37–40.

Haxo, F. T., and L. R. Blinks, 1950. Photosynthetic action spectra of marine algae. *J. Gen. Physiol.*, **33**: 389–422.

Pirson, A., 1957. Induced periodicity of photosynthesis and respiration in *Hydrodictyon*. *In:* Research in Photosynthesis. Edit. by H. Gaffron *et al.*, pp. 490–499. New York: Interscience Publishers, Inc.

Shimada, B. M., 1958. Diurnal fluctuation in photosynthetic rate and chlorophyll "a" content of phytoplankton from Eastern Pacific waters. *Limnol. Oceanogr.*, **3**: 336–339.

Sorokin, C., 1960. Photosynthetic activity in synchronized cultures of algae and its dependence on light intensity. *Arkiv f. Mikrobiol.*, **37**: 151–160.

Tamiya, H., 1957. Mass culture of algae. *Ann. Rev. Plant Physiol.*, **8**: 309–334.

Yentsch, C. S., and J. H. Ryther, 1957. Short-term variations in phytoplankton chlorophyll and their significance. *Limnol. Oceanogr.*, **2**: 140–142.

CONTROL OF FLASHING IN FIREFLIES. I. THE LANTERN AS A NEUROEFFECTOR ORGAN

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The ability of some fireflies to produce remarkably uniform and brief flashes of light is of much interest in relation to cellular control mechanisms. Neural involvement has been implicit or explicit in most theories of flash regulation, but there are two main ideas as to its ultimate mechanism. According to one view the luminescence of the photocyte is controlled by limiting the access of oxygen by supposed mechanical valves (tracheal end cells). According to the other view the terminal nerves stimulate the photocyte directly. The oxygen theory has been dealt with in previous papers (Buck, 1948; Hastings and Buck, 1956). The present series of papers explores responses to electrical stimulation.

Work by Macartney (1810), Macaire (1821), Todd (1826), Joseph (1854), Kölliker (1858), Owsjannikow (1868), Bellesme (1880), Arnold (1881), Verworn (1892), Perkins (1931), Brown and King (1931), Snell (1932), Alexander (1943) and others showed that voluntary luminescence is abolished by decapitation and can be elicited by electrical stimulation. Dubois (1886), Heinemann (1886), Fuchs (1891), Lund (1911) and Gerretsen (1922) attempted to localize the stimulation to specific efferent nerves, though at a very crude level. Steinach (1908) reported evidence of summation of subliminal stimuli, and Chang (1956) recorded facilitated series of responses from both the larval and adult firefly and compared them with responses in muscle. Hanson (1961) showed that regional excitation corresponds to the gross innervation of the lantern, and Carlson (1961) found that even the "pseudoflash," caused by passive entry of oxygen into an hypoxic lantern, is affected by prior neural stimulation. Nonetheless, the type and degree of neural involvement in flash control remain uncertain because of ignorance of where the nerves in the lantern terminate. Thus, nerves might control either end cells (oxygen control) or photocytes directly, and the hypoxic effect might be a direct limitation or, as Brücke (1881) suggested long ago, an inactivation of control nerves.

In view of this impasse it seemed possible that understanding of the role of nerve in triggering the firefly flash might be gained by analyzing the flash as an effector event and exploring its variation with changing stimulus parameters. Subsequent papers in this series will deal with central nervous aspects of excitation, peripheral nervous phenomena, and effector unit response. Preliminary summaries of some of the work have been given (Case and Buck, 1957, 1958, 1959a, 1959b).

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Materials and Methods

The fireflies investigated were adults of *Photinus pyralis* (Linné) from Maryland and Iowa, *Photinus marginellus* Le Conte and *Photinus consanguineus* Le Conte from Woods Hole, *Photinus punctulatus* Le Conte from Iowa, and both larvae and adults of the most common photurid (tentatively identified as *Photuris versicolor* Barber) from each of the three localities. (Identifications were made via the papers of Barber and McDermott (1951) and Green (1956).) Adult specimens were netted in the field and used within a week, meanwhile being kept unfed in humidified jars at room temperature. Larvae were collected on lawns near streams and kept in petri dishes on moist paper, either at room temperature for immediate use or at 5–10° C. for use weeks or months later.

Preparations included intact individuals, decapitated specimens, and isolated lanterns. To prepare the adult lantern, which consists of ventral plaques in abdominal segments 6 and 7, these segments were excised and all viscera overlying the photogenic organ, usually including the terminal ganglia of the ventral nerve cord, were removed. The larval light organs, a pair of small oval spots on the ventrolateral surface of abdominal sternite 8, were excised with part of the surrounding sternite.

Stimuli were delivered by Grass S-4 stimulators via radio-frequency isolation units and electrodes of unshielded 30-gauge silver or platinum-iridium wire. Intact or decapitated specimens were de-legged and anchored on dental wax with wire staples spanning the body transversely. Isolated light organs were laid across a parallel electrode pair in a small moist chamber.

Two methods were used in temperature experiments. In one, the specimen, with electrodes in position, was bound to the bulb of a thermometer reading to 0.01° suspended in air in a test tube immersed in a water bath; in the other the specimen was attached to a thermistor thermometer readable to 0.5°, in a small copper air chamber through the walls of which water was circulated from a large temperature-stabilized reservoir.

Light emission was followed with an RCA 931-A photomultiplier leading directly to one channel of a dual beam oscilloscope and photographed simultaneously with a second trace carrying a stimulus marker.

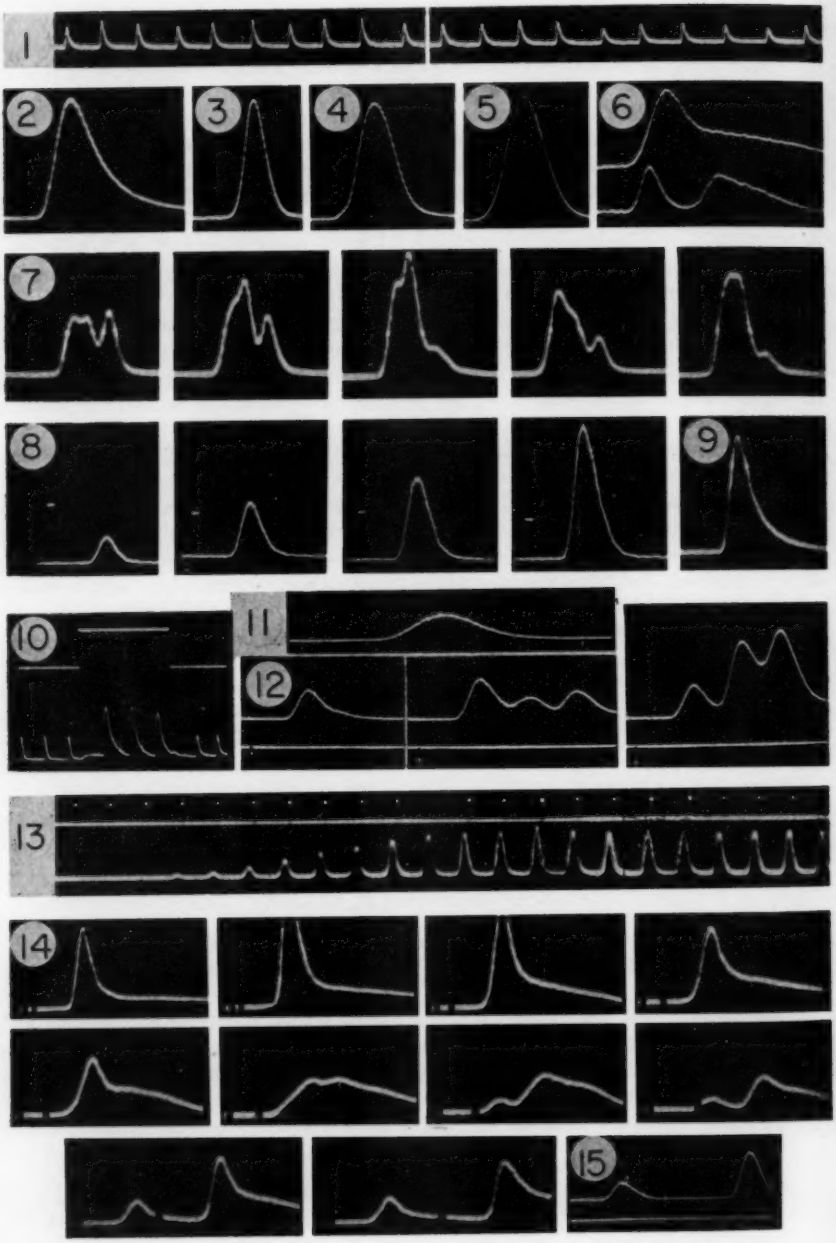
In all except the temperature experiments the preparation was mounted on the stage of a stereoscopic microscope magnifying up to 120×. Unless otherwise specified, all experiments were done at 25 ± 3° C.

Results

Both spontaneous and induced flashing exhibit a high degree of consistency, and most of the illustrations and data presented are typical of responses obtained in dozens or hundreds of measurements. Instances of refractoriness associated with apparent central nervous phenomena will be considered in the following paper.

1. *The spontaneous flash*

Spontaneous flashing is seen only in individuals with brain still connected with cord. As a general rule it does not occur in either field or laboratory below 18–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20° C. Under the conditions of most of our experiments (animal immobilized; electrodes inserted), spontaneous flashes were generally infrequent, although single responses could sometimes be induced by various types of mechanical stimulation. *Photuris* was exceptional in its spontaneous activity, some specimens flashing with almost clock-like regularity. Figure 1 shows the first 10 and last 10 of 80 consecutive flashes given by a male of *Photuris* in 32 seconds. In this series the mean interflash interval was 405 ± 3.2 milliseconds (S.E. = 0.8% of mean) and mean flash intensity was 21.3 ± 0.5 arbitrary light units (S.E. = 2.4% of mean).

Insofar as flash kinetics are concerned, oscilloscopic observation confirms field impressions in showing characteristic differences between species. Thus, the durations of flashes of comparable magnitude may vary from 200 mscs. or less in *Photuris* (Fig. 9) to a second or more in *Photinus pyralis* (Fig. 2). Single flashes may have nearly symmetrical accretion and decay phases (Figs. 3, 4, 5) or show marked skewing (Figs. 2, 9). Flashes may be either single or multiple (Figs. 6, 7). We have already emphasized the usual constancy of flash type within a species (Figs. 1, 13, 32 etc.) but it is also relevant to the control problem to point out differences that may occur between the flashes of the two sexes of one species (Figs. 6 *vs.* 3 *vs.* 9), between different individuals of the same sex and species (Figs. 3 *vs.* 9; 6), and even between successive flashes of one individual (Fig. 7). Sometimes these variants have ready explanations, such as differences in degree of disturbance of the specimens, or asynchrony or variable involvements of different lantern regions; but some are not presently understood.

In most species the spontaneous flash under laboratory conditions appears comparable to that emitted in natural flight. However, in *Photuris*, which is a variable and confusing genus even in field behavior (Barber and McDermott, 1951), the free-flying male of the Woods Hole variety typically gives a rapid four-peaked twinkle every second or two, whereas the female gives a long lingering glow at irregular intervals; in the laboratory both sexes usually give single flashes, although

FIGURES 1–15. Note that in photos of oscilloscope traces the time scale (S) is given as the width of the print. Some stimulus artifacts have been retouched. (1) Woods Hole *Photuris*, male. First and last 10 of a series of 80 spontaneous flashes. S = 8 sec. (2) Maryland *Photinus pyralis*, male. Spontaneous flash. Light did not regain base level for 1 second. S = 735 mscs. (3) Woods Hole *Photuris*, male. Spontaneous flash. S = 220 mscs. (4) *Photinus marginellus*, male. Spontaneous flash. S = 450 mscs. (5) Maryland *Photuris*, male. Spontaneous flash. S = 130 mscs. (6) Woods Hole *Photuris*, female. Two frames of spontaneous flash of two individuals. S = 300 mscs. (7) *Photinus consanguineus*, male. Five successive spontaneous flashes. S = 640 mscs. (8) Woods Hole *Photuris*, male. Responses of isolated lantern to 5, 7, 8 and 9 volts, all at 10 mscs. S = 200 mscs. (9) Woods Hole *Photuris*, male. Spontaneous flash. S = 240 mscs. (10) Woods Hole *Photuris*, female, decapitated, electrodes in light organ. Eight successive responses to 10 mscs./15 v. with the fourth, fifth and sixth occurring in a 4 v. D.C. field. S = 10.5 secs. (11) *Photuris* larva, decapitated, electrodes immediately anterior and posterior to light organ. Induced glow, 10 mscs./15 v. S = 3.6 secs. (12) *Photinus punctulatus*, female, isolated lantern. Three frames showing responses to 4, 8 and 10 v./4 mscs. S = 350, 500, 500 mscs. (13) Woods Hole *Photuris*, sex unknown, decapitated, electrodes in lantern. Response to stimulus train of 4 mscs./5 v. at 10 per second. S = 2.2 secs. (14) Woods Hole *Photuris*, female, decapitated, electrodes in lantern. Responses to paired stimuli of 6 mscs./15 v. with intervals of 15, 20, 30, 40, 50, 60, 70, 90, 150 and 200 mscs. S = 360 mscs. except 430 mscs. for last two frames. Stimulus indicated by break in photomultiplier trace. (15) Maryland *Photinus pyralis*, male, decapitated, electrodes immediately anterior and posterior to lantern. Responses to two equal stimuli 1200 mscs. apart. S = 2 secs.

at least the female occasionally produces compound flashes (Fig. 6) as does the female of the Maryland *Photuris* (Fig. 8 of Hastings and Buck).

In larvae of most lampyrid fireflies the photogenic organ is much smaller and simpler than the adult lantern, and light is emitted as a long glow rather than a short flash. No spontaneous glows were recorded, because of their very irregular timing and low frequency, but the kinetic contrast between larval and adult luminescence is well illustrated by the induced glow (Fig. 11; note time scale). Actually, the duration of the larval glow is usually even longer in nature—often several seconds—but in view of our observations at different temperatures (see below) this is quite possibly accounted for by the temperature of the soil surface being considerably below 25°, particularly on the damp evenings in early autumn when larvae are most easily observed.

2. *Induced flashing : general*

Although, as will be shown, it is possible to alter flash duration, intensity and form by changing stimulus parameters, a single, moderate electric shock usually elicits a flash indistinguishable from the normal spontaneous flash characteristic of the species. Seemingly normal flashes can result with the electrode pair at any level of the body from head to lantern, but are usually most reliably produced with ab-

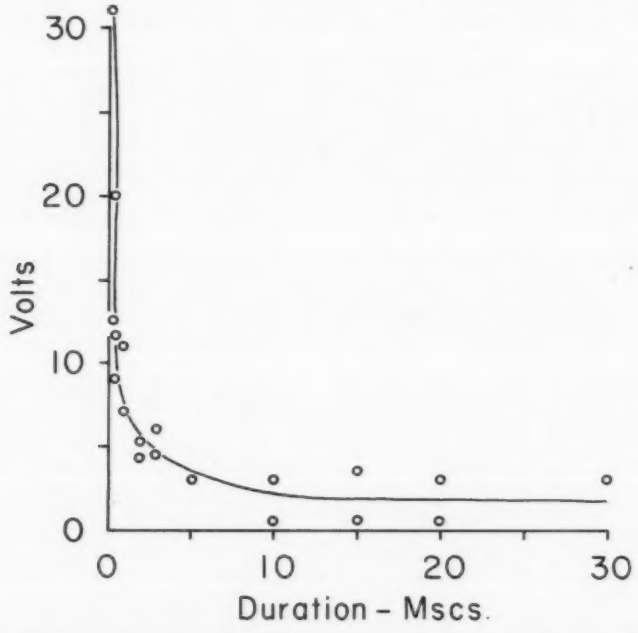


FIGURE 16. Representative strength-duration curve for minimum visually detectable response induced by direct stimulation of lantern of a decapitated Woods Hole *Photuris* male.

dominal stimulation. In the observations described below, induced responses, unless specifically excepted, were recorded with electrodes either in abdominal segment 6 at the level of the last ganglionic mass of the cord, or actually inserted in lantern tissue. In both instances excitation is presumably *via* peripheral nerve.

In connection with changes in flash characteristics, it is important to avoid being misled by differences due simply to magnitude. In Figure 39, for example, the weakest and strongest flashes might be thought to be quite different in form, whereas in fact the relative rates of rise and fall in light intensity are the same in both, and the two flashes could be made to coincide by equalizing their peaks.

3. *Threshold*

The isolated light organ, 15 minutes after preparation, gives reproducible threshold values apparently not much affected by unavoidable variations in tissue-electrode contact. Using this preparation, the interrelations of stimulus voltage and duration for minimum detectible response were studied. The results (Fig. 16) show strength-duration relations similar to insect nerve-muscle. In seven males of *Photuris* with electrodes in the lantern the average rheobase was 2.1 volts and the chronaxie was 3.9 mscs. Minimum values were 0.7 volts and 1.7 mscs., respectively. Values for the female of *Photuris* and for males of *Photinus pyralis* and *P. punctulatus* were in the same range as for the male of *Photuris*.

Threshold for direct current stimulation is relatively uniform. In *Photinus pyralis*, for example, the values in three individuals were 5.5–6.0, 3.5–4.0 and 7.5–8.0 volts.

Threshold is much increased at lower temperatures. In typical measurements on *Photuris* a 10 mscs./6 v. shock elicited a flash of good intensity at 25°, whereas, in the same individual, 50–60 volts at the same duration were required in the 9–12° range to obtain even a much weaker response.

4. *Factors affecting flash intensity*

a. *Stimulus strength and duration.* Within limits, flash intensity varies with voltage of single shocks of given duration (Figs. 8, 17). The same effect is seen during stimulation imposed upon D.C. fields (Fig. 10). In certain species, particularly if the lantern still has central connections, increasing strength of stimulus may induce multiple flashes (Fig. 12). These probably autoexcitatory responses will be considered in detail in the second paper of this series.

As would be expected from the strength-duration findings, variation in stimulus duration alone also affects flash intensity. These effects are considered in the section on flash form.

b. *Effects of stimulation frequency on flash intensity: facilitation.* With subliminal stimulation, the adult firefly lantern exhibits typical summation, response first occurring after several shocks and then growing (Figs. 13, 44). Additive effects occurring with supra-threshold stimuli are well displayed by paired shocks (Figs. 14, 18). The minimum delay which produced detectible summation (*i.e.*, the absolute refractory period) was not investigated in detail, but is clearly less than 5 mscs. in *Photuris* and is apparently between 6 and 8 mscs. in *Photinus pyralis*, using near-threshold stimuli in both instances. Above these minima, facilitation

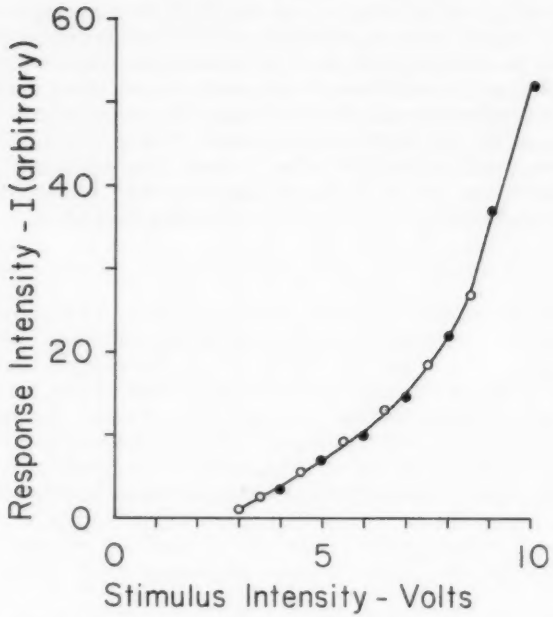


FIGURE 17. Relationship between stimulus strength and flash intensity at constant 10 mscs. stimulus duration. Open circles represent ascending voltage series, closed represent descending series. Woods Hole *Photuris* male, isolated lantern.

increases rapidly to a maximum that shows considerable variation both intra- and inter-specifically. In six specimens of *Photuris* maximum facilitation occurred with a stimulus interval of 10–20 mscs. (Fig. 18), but in two other individuals the most effective intershock interval seemed to be at least 30 mscs. In most specimens of *Photinus pyralis* the buildup and decay of facilitation was more gradual, the most effective delay lying between 50 and 100 mscs.

After maximum facilitation is attained, flash intensity declines sharply with increasing delay. In *Photuris* the facilitating effect of the second shock is practically dissipated by the time the delay reaches 60 mscs.

Concurrently with changes in flash intensity, increasing intershock delay causes changes in flash form, manifested by a slowed rate of rise without change in latency —*i.e.*, a shift in peak position toward later time (Fig. 14, first *vs.* fifth frames), relegation of the original flash to a shoulder on the rise phase of the second response (Fig. 14, sixth and seventh frames), and finally by a splitting of the flash into two peaks representing the separate responses to the two shocks (Fig. 14, eighth and ninth frames). In *Photuris* the separation is complete at an inter-stimulus interval of approximately 85 mscs: in *Photinus pyralis* not until 300 mscs.

At the interval for which separate responses to paired shocks are first detectible, the first flash is sometimes the more intense. However, as the peaks draw apart

the second usually becomes larger than the first (Fig. 14, frame seven *et seq.*). In both *Photuris* and *Photinus pyralis* this apparent facilitation of one response by the preceding one persists, in some instances, for more than a second (Fig. 15).

Facilitation is also shown by the *Photuris* larva, according to data collected by Dr. Albert Carlson (Fig. 19). Here the maximal summation effect of the second stimulus on the response to the first is reached only after 50–60 mscs., and persists at least 500 mscs., a much longer time than in the adult. The augmentation of the second response of a pair as a function of the first response was not investigated in larvae, but, if comparable to other adult/larval response rate ratios, would be expected to persist at least 10 seconds.

When repetitive stimulation is extended from paired shocks to trains, response varies widely depending on stimulus frequency and voltage, temperature, degree of neural integrity, degree of facilitation, and species. Figures 13 and 22 illustrate the rather stable sort of response seen typically with moderate stimulation, in which each flash is independent and the responses increase progressively in intensity and duration for several successive stimulation intervals, then reaching a plateau. As might be expected, the gain in flash intensity is usually more rapid the higher the rate of stimulation (Figs. 20, 21), and in species or individuals producing double flashes, both flash components augment (Fig. 22).

The stimulation frequency limit for production of completely or partially independent responses of course varies with flash duration, among other factors. In

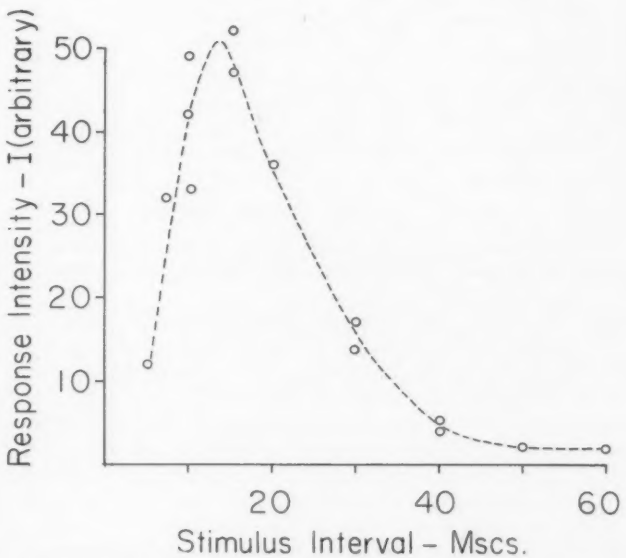


FIGURE 18. Temporal facilitation of electrically induced flashes. Abscissa represents interval from first to second stimulus of a pair, in all instances 3 mscs./5 v. Woods Hole *Photuris* female, isolated lantern. Typical of five other specimens.

Photuris, completely separate responses to each stimulus of a train can usually be elicited at frequencies of at least 10/sec. (Fig. 13), whereas in *Photinus pyralis* any frequency higher than about one per second prevents the luminescence from falling to baseline before the next response. As stimulation frequency is still further increased the individual flashes necessarily begin to merge, the fusion giving a maintained luminescence that changes more or less in proportion to flash intensity, and, in combination with facilitation, may produce responses quite similar to the familiar staircase and tetany of striated muscle (Figs. 23, 24). However, it is not uncommon, when close to the limit of 1 : 1 response to continued serial stimulation, to find the average response intensity falling off markedly after a brief rise (Figs. 25, 27, 30, 33).

Species differences are strikingly illustrated in ability to follow the stimulation frequency. The Woods Hole photurid, for example, is able to respond 1 : 1 up to about 20 shocks per second (Fig. 27). The Iowa photurid fails at between 10 and

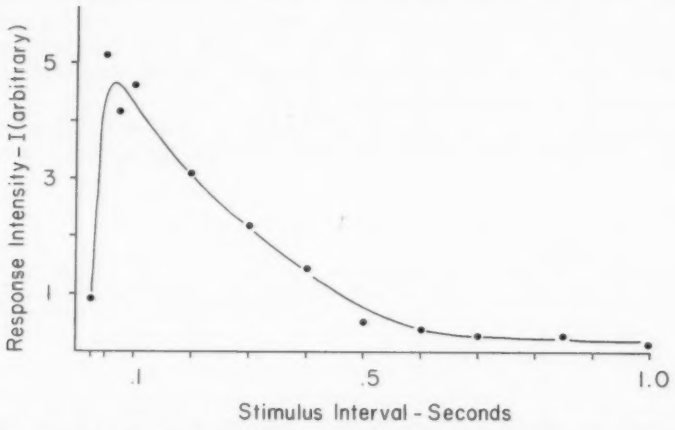


FIGURE 19. Temporal facilitation of electrically induced flashes in the larval firefly. Abscissa as in Figure 18. Stimulus 10 mscs./4 v. Iowa *Photuris* larva, isolated light organ.

15 per second (Fig. 33), *Photinus punctulatus* reaches its limit at about 10/sec. (Fig. 23), the Iowa *P. pyralis* (Fig. 31) and *P. marginellus* at 7/sec., while the Maryland *P. pyralis* barely follows at 4/sec. even with intense stimuli.

Up to the species limit, the ability to respond 1 : 1 to a particular frequency of stimulation increases with either increasing voltage or increasing stimulus duration. The actual frequency limit shows a definite relation to the strength-duration product in that 1 : 1 response can be maintained by a variety of duration-voltage combinations. In *P. marginellus,* for example, the frequency limit of about 7/sec. applies at stimuli ranging from 50 mscs./3 v. to 3 mscs./50 v. However, neither near the limit of 1 : 1 response nor at more moderate frequencies does there appear to be a true reciprocity in the response itself, *i.e.,* in mean flash intensity (Fig. 32).

An effect commonly seen at the frequency response limit is a rather regular alternation in flash intensity (Figs. 28, 30, 33). At stimulation frequencies exceed-

ing the limit of 1 : 1 response, the response differs somewhat depending on species, on stimulus parameters, and on whether or not the lantern is deganglionated, but generally resembles either a more or less typical tetany (Figs. 24, 25) or consists of serial responses to separate stimuli at some exact fraction of the actual stimulation frequency (Figs. 31, 34, 35).

d. *Adaptation and fatigue.* Free-flying fireflies can produce many dozens of consecutive flashes at the species-characteristic frequency without apparent fatigue. Even when flashing excitedly in the laboratory at much higher than normal frequencies, little decrement in either frequency or brightness of spontaneous flashes can be detected (there is about a 20% falloff in mean peak height and a 6% lengthening of interflash interval between the two parts of Figure 1). Repetitive driven flashing may, depending on the conditions of stimulation, either continue practically unchanged for long periods (Fig. 13), go through cycles of varying excitability (Figs. 21, 26, 32), or cease relatively quickly. As an example of one extreme, a female *Photuris* stimulated once per second flashed for more than 70 minutes with only slowly decreasing vigor (over 4000 successive flashes). At the other extreme

TABLE I

Stimulus-response interval at 25° C. Two to six measurements per individual

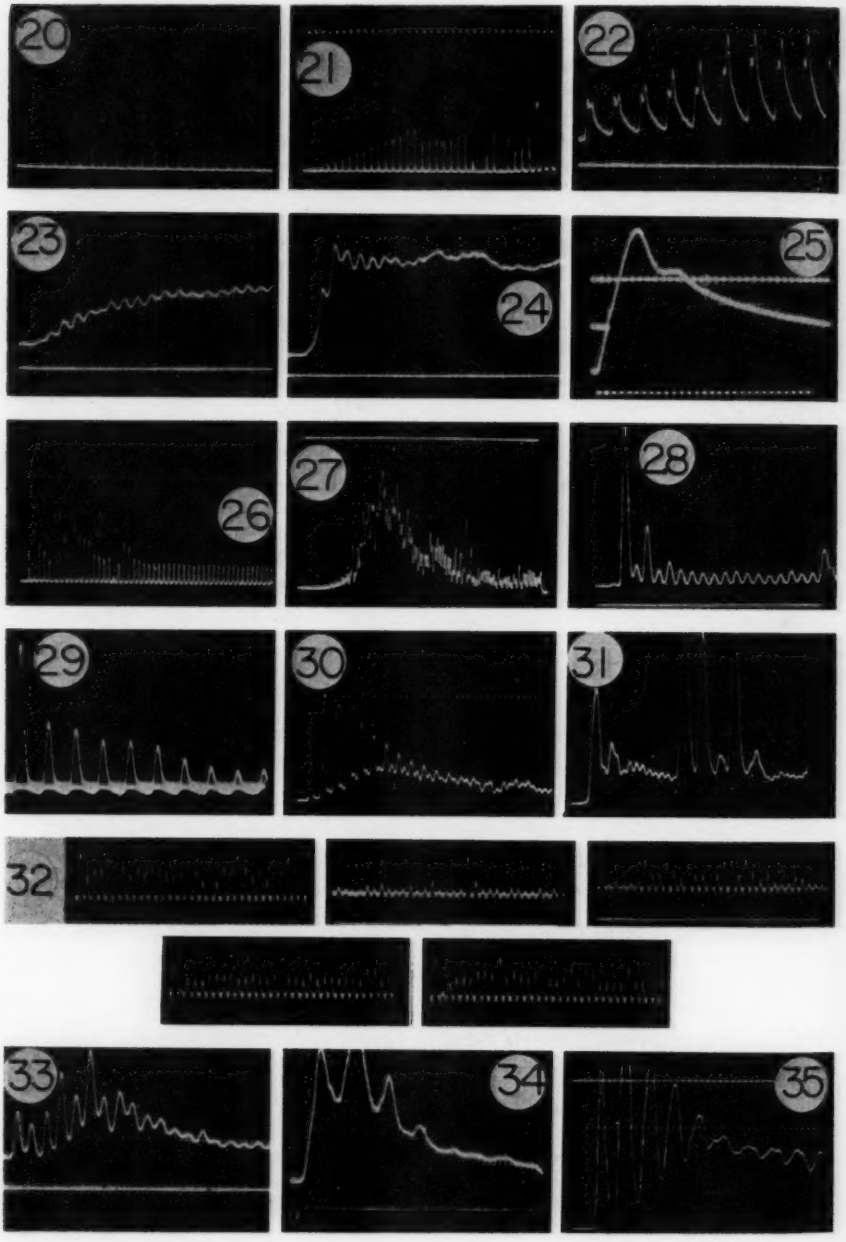
Species	No. individs.	Latency (milliseconds)	
		Average	Range
Photuris	6	86	55–101
Photinus pyralis			
Maryland	4	194	160–206
Iowa	4	166	144–190
Photinus punctulatus, male	10	67	50–85
Photinus punctulatus, female	2		125, 125
Photinus marginellus	2		26, 27
Photinus consanguineus	8	Approx. 100, 175, 250 (3 elements)	
Photuris, larva	1	800	

are records of the sort illustrated in Figures 29, 30 and 38 in which the response decreased greatly or even failed after a few stimuli. Such damping is particularly common when stimulation frequency exceeds the limit of 1 : 1 response (Fig. 34). Rapid degradation in flash intensity in 1 : 1 response series can probably be ascribed to junctional fatigue when shock voltage is high, and to adaptation when stimulus strength is little above threshold.

In addition to frequency effects already described, it was noted that the first response to a train was sometimes conspicuously more intense than the succeeding flashes (Figs. 29, 36, 37, 63, 64).

5. *Latency*

Table I gives a sample of stimulus-response delays from among some hundreds of flashes of good intensity measured in adult fireflies of several species at about 22° C. under comparable conditions of stimulation. These data show that there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may be significant and characteristic latency differences between species, or even possibly between what appear to be regional variants of one species (*P. pyralis*). The considerable latency difference between the sexes of *P. punctulatus* was not found in *Photuris*, and females of the other species are so seldom found that no measurements were obtained.

Considering the probable multiplicity of the excitation pathways and the number of response units in the lantern, response latency within individuals is remarkably constant. Serial responses of comparable intensity given in response to regularly repeated identical stimuli may vary less than 5% of their mean value, particularly in deganglionated lanterns.

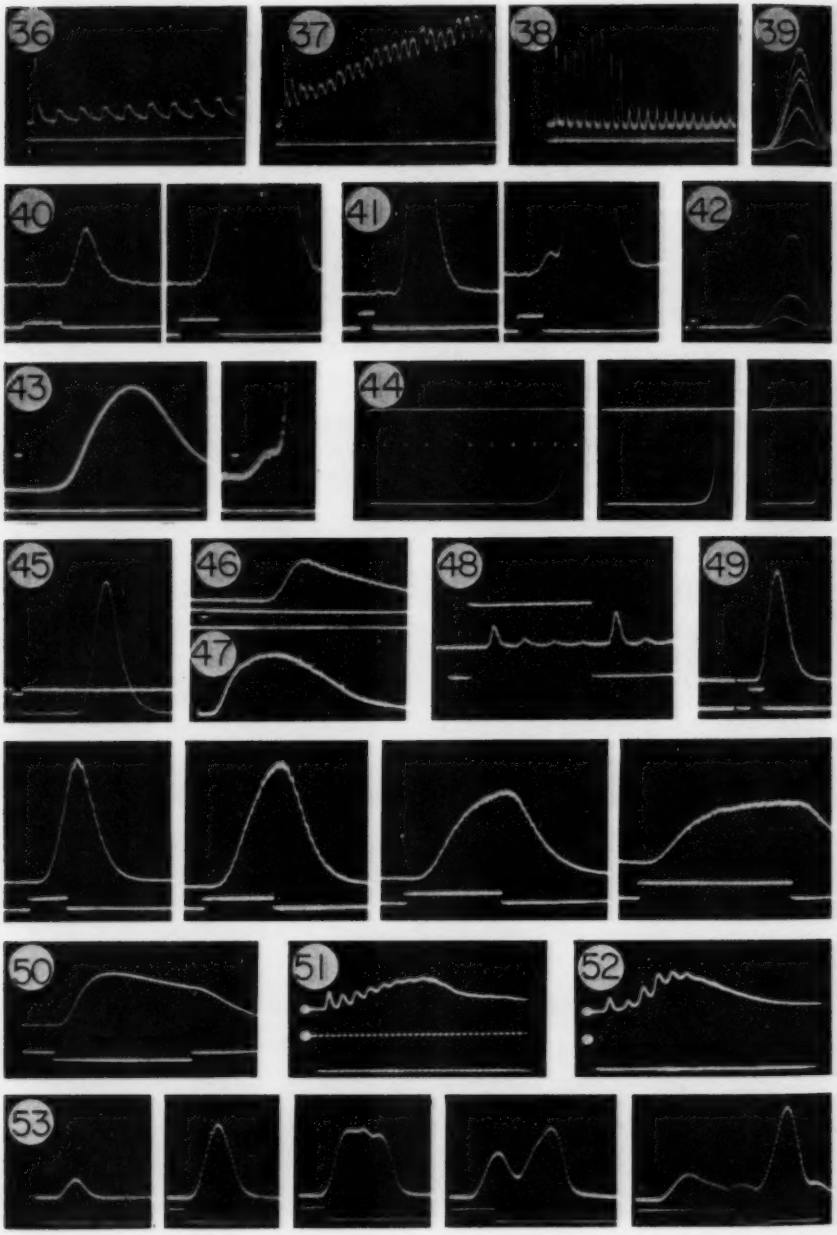
a. *Effects of temperature on latency.* In *Photuris* and in *Photinus pyralis*, our most used species, 8° C. seems to be about the limit to which temperature can be lowered and still permit induced flashing, and even this cannot be accomplished without strong stimulation (see Threshold), change in form (see below), and much prolonged latency (Fig. 54). Above this temperature, latency decreases, at first rapidly. Between 32° C. and 35° C. in *Photuris* there is some indication of an inflection point. In a male studied (not plotted) it was particularly striking. Below this point the temperature-induced changes in latency are fully reversible, but above it permanent damage seems to occur, as indicated by failure of the latency curve to retrace its path as temperature is lowered again.

Since overall latency reflects a complex of conduction and excitation steps, mostly unknown, Q_{10} values are of uncertain worth; however, they lie within the usual range for neuroeffector systems (*ca.* 1.5 at 30° C., and over 2.0 at 20° C.). Temperature characteristics are of even more tenuous significance, but the data appear to yield linear Arrhenius plots up to 30° C.

Though measurements of latency changes of the larval "flash" with temperature are available only for the 14°–26° C. range, differences from the adult pattern are suggested (Fig. 55). First, the trend seems to be linear rather than inflected. Second, the Q_{10} is about 3.0, or about 50% higher than the value for the adult in the corresponding range.

FIGURES 20–31. (*20*) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Responses to 10 mscs./15 v. at 2 per second. S = 11 secs. (*21*) Same as Figure *20* except stimulus frequency 7 per second. S = 5.5 secs. (*22*) *Photinus punctulatus*, male, isolated lantern. Responses to 2 mscs./10 v., at 2 per second. S = 5 secs. (*23*) Same as Figure *22* except stimulus frequency 10 per second. S = 2 secs. (*24*) Same as Figure *22* except stimulus frequency 20 per second. S = 2.1 secs. (*25*) Iowa *Photinus pyralis*, male, decapitated, electrodes in lantern. Responses to 40 mscs./150 v. at 15 per second. S = 2.5 secs. (*26*) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Stimulus 5 mscs./15 v. at 10 per second. S = 5.7 secs. (*27*) Same as Figure *26* except stimulus frequency 20 per second. S = 5.5 secs. (*28*) Iowa *Photinus pyralis*, male, decapitated, electrodes in lantern. Stimulus 5 mscs./10 v. at 4 per second. S = 5.5 secs. (*29*) Iowa *Photuris*, male, decapitated, electrodes in lantern. Stimulus 1 msc./6 v. at 5 per second. S = 2 secs. (*30*) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Stimulus 5 mscs./15 v. at 20 per second. S = 2.1 secs. (*31*) Same as Figure *28* except stimulus frequency 7 per second. S = 5.5 secs.

FIGURES 32–35. (*32*) *Photinus marginellus*, male, decapitated, electrodes in lantern. Responses to five series of stimuli at 3 per second: 12 mscs./5 v., 20 mscs./3 v., 6 mscs./10 v., 4 mscs./15 v., and 2 mscs./30 v. S = 1 sec. (*33*) Iowa *Photuris*, male, isolated lantern. Stimulus 2 mscs./20 v. at 10 per second. S = 2 secs. (*34*) Same as Figure *33* except stimulus frequency 20 per second. S = 2 secs. (*35*) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Stimulus 5 mscs./15 v. at 40 per second. S = 2.1 secs.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b. *Effects upon latency of stimulus strength, duration and frequency.* Latency rather frequently appears to decrease progressively with increasing strength of stimulus (Figs. 8, 40). In many instances these effects can be ascribed to the magnitude artifact already mentioned, and this is probably also the explanation of the apparent latency changes seen in responses facilitated in serial stimulation (Figs. 39, 42). Latency may also appear to decrease stepwise due to the becoming visible of an additional response of shorter latency. Such early shoulders on the main flash may appear simply as a consequence of increased amplification of the photomultiplier trace (Fig. 43), or be invoked by increasing stimulus strength (Fig. 40) or duration (Fig. 41).

Striking differences in ostensible latency may occur with trains of subliminal stimuli which exceed the capacity of the lantern for 1:1 response (Fig. 44), although there is ambiguity as to which is the first effective stimulus.

With strong stimulation, some instances of shortened latency are due to the evocation of a separate and qualitatively different sort of early response ("quick flash"—Case and Buck, 1958; 1959b) that is considered in detail in the third paper of this series.

6. *Flash form*

Flash form—the variation in light intensity with time—is subject to so many internal and external influences that few generalizations are possible. We have already alluded to changes in form involved in the transition from single to double flashes (Fig. 14), and similar changes are seen in the evocation of multiple responses by increased stimulus voltage (Fig. 12) and duration (Fig. 53), as well as in spontaneous flashing (Fig. 7). Some factors influence differently the rates of

FIGURES 36–53. (36) *Photinus punctulatus*, male, isolated lantern. Stimulus 2 mscs./10 v. at 2 per second. S = 5 secs. (37) Same as Figure 36 except stimulus frequency 5 per second. S = 5 secs. (38) Iowa *Photuris*, male, isolated lantern. Stimulus 1 msc./5 v. at 5 per second. S = 5.5 secs. (39) Woods Hole *Photuris*, female, decapitated, electrodes immediately anterior and posterior to lantern. Five superimposed responses to 6 mscs./20 v. Temperature 17.9° C. S = 80 mscs. (40) Woods Hole *Photuris*, male, decapitated, electrodes in lantern. Stimulus 60 mscs./4 v. and 60 mscs./15 v. S = 250 mscs. (41) Same as Figure 40 except stimuli 20 mscs./15 v. and 40 mscs./15 v. S = 250 mscs. (42) Iowa *Photuris*, male, isolated lantern. Stimulus 6 mscs./20 v. Temperature 26.7° C. S = 190 mscs. (43) *Photinus consanguineus*, male, isolated lantern with intact ganglia. Two responses to 10 mscs./15 v., the second at 12.5 times the amplification of the first. S = 480, 230 mscs. (44) Maryland *Photinus pyralis*, male, decapitated, electrodes on prothoracic ganglion. Responses to 2 mscs./9 v. at 10, 20 and 40 per second. S = 1100, 650, 440 mscs. (45) Woods Hole *Photuris*, female, decapitated, electrodes in lantern. Stimulus 10 mscs./6 v. Temperature 25.5° C. S = 160 mscs. (46) Same as Figure 45 except stimulus 10 mscs./80 v. Temperature 12° C. S = 600 mscs. (47) Woods Hole *Photuris*, female, decapitated, electrodes in lantern. Stimulus 10 mscs./40 v. Temperature 39.2° C. S = 500 mscs. (48) Woods Hole *Photuris*, male, isolated lantern. Stimulus 400 mscs./8 v. S = 860 mscs. (49) *Photinus consanguineus*, male, isolated lantern. Stimuli 30 mscs./15 v.; 100 mscs./10 v.; 200 mscs./9.5 v.; 300 mscs./9.5 v.; 500 mscs./12 v. S = 250, 306, 340, 410, 390 mscs. (50) *Photinus marginellus*, male, isolated lantern. Stimulus 500 mscs./90 v. S = 850 mscs. (51) Woods Hole *Photuris*, female, decapitated, electrodes in thorax. Stimulus 20 mscs./15 v. at 30 per second. S = 1.4 secs. (52) Same as Figure 51 except stimulus 12 v. D.C. beginning with deflection of lower trace. S = 1.4 secs. (53) *Photinus marginellus*, male, decapitated, electrodes in light organ. Stimuli 15 v. for 20, 80, 100, 200, 500 mscs. S = 800, 650, 700, 950, 1100 mscs.

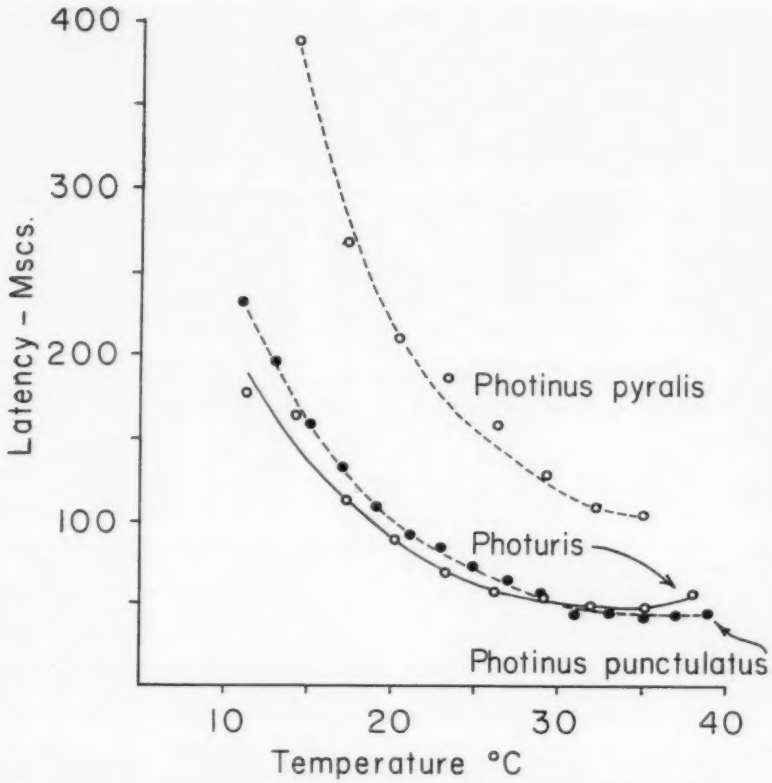


FIGURE 54. Stimulus to response latency in relation to temperature for adults of three species of fireflies. Stimulus varied as function of temperature in order to produce similar magnitude flashes. Points represent means of measurements on 5 male *P. pyralis*, 10 female Woods Hole *Photuris*, 6 male *Photinus punctulatus*. Direct excitation of lantern.

accretion and decay of light intensity. One of the most predictable of these is temperature. Between approximately 15° and 30° C. flash form remains relatively constant (Fig. 56), but temperatures outside this range usually produce a preferential slowing of the decay phase of the flash (Fig. 45 *vs.* Fig. 46). This slowing is usually progressively greater the more extreme the temperature, but in some series there appears to be a relatively sudden form change at around 15° C. The changes toward the ends of the range are difficult to evaluate quantitatively because of the developing steady glow (see below) and the necessarily concomitant changes in stimulus strength. The low temperature skewing seems to be completely reversible, but the slowed decay at high temperature (Fig. 47) persists after return of the animal to normal temperatures, suggesting irreversible damage.

Flash form is of course affected by long or high frequency stimuli that prolong

luminescence. Several such deviations can be induced. A curious effect of lengthening shock duration is a slowed rise demonstrable in flashes of comparable peak intensity given by deganglionated lanterns (Fig. 49). There is also often a perceptible "off effect," of the same latency as the primary rise (Figs. 48, 53). However, it is also clear that the lantern can be stimulated throughout the duration of long stimuli, since a flash of many times normal duration can be produced (Figs. 49, 50). Presumably the same explanation applies to the fact that trains of stimuli above the frequency limit of 1:1 response (Fig. 51) may produce the same effect as the continuous passage of current (Fig. 52).

There seem clearly to be elements of both facilitation and of fatigue or adaptation in artificially lengthened flashes. Depending on whether the voltage is low or high, shocks of equal duration may induce either flashes which increase in intensity up to the break point (Fig. 49, fifth frame) or which start high and decline steadily (Fig. 50).

Customarily the peak positions in a homologous series of flashes coincide rather closely (Figs. 39, 57), indicating that the *rate* of rise of light intensity is independent of total light production. However, in some series the peak positions of

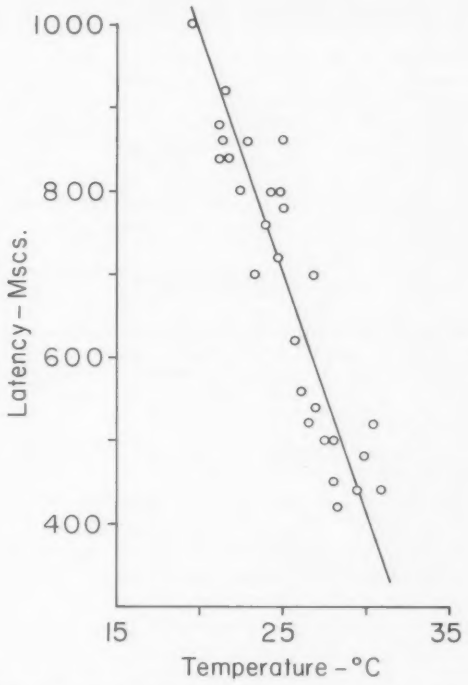


FIGURE 55. Stimulus to response latency for Iowa *Photuris* larvae. Stimulus strength varied with temperature to produce similar magnitude flashes.

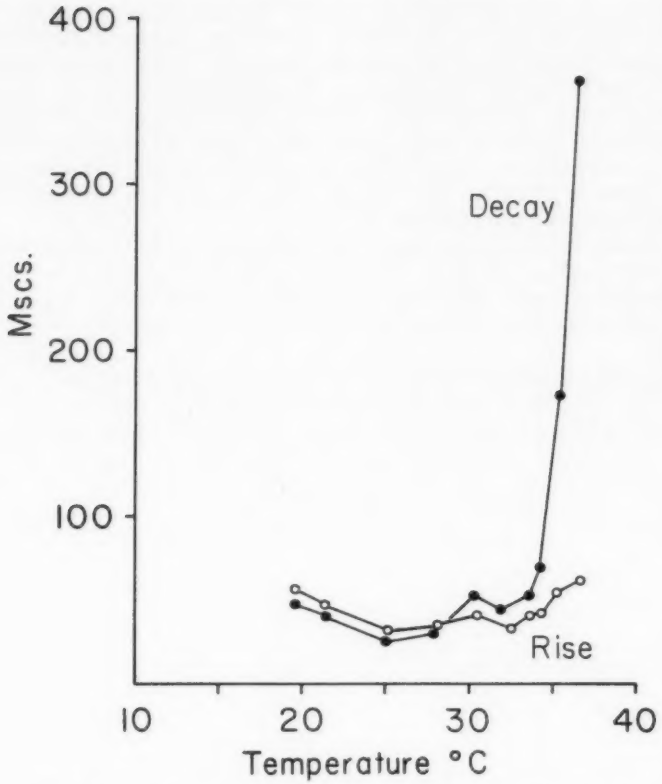


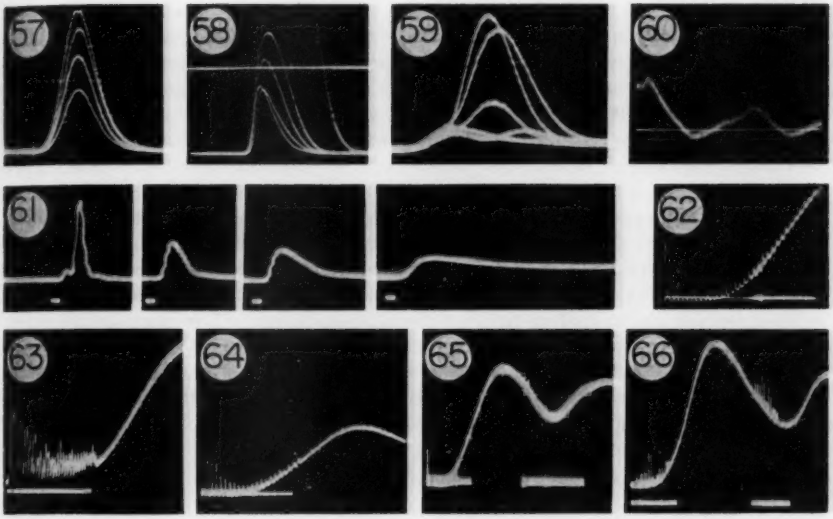
FIGURE 56. Influence of temperature on flash form as indicated by variation in half-rise and half-decay times of flashes induced by electrical excitation of lantern. Stimulus strength varied with temperature in order to produce as nearly equal magnitude flashes as possible. Iowa *Photuris* male.

the low intensity flashes are variable, usually, but not always, in the direction of early peaking (Figs. 58, 59).

Numerous other interesting alterations in flash form of even more obscure causation have been observed. When, for example, a firefly is stimulated every few seconds in decreasing ambient oxygen concentration the decay phase of the flash sometimes tends to be slowed much more than the rise phase as hypoxia deepens (Fig. 61).

7. Glow

In addition to the various types of more or less brief flash, all species of firefly may show moderate to long-continuing steady luminescences which vary widely in



FIGURES 57–66. (57) Woods Hole *Photuris,* female, decapitated, electrodes in prothoracic ganglion. Four superimposed responses to 10 mscs./10 v. S = 100 mscs. (58) Woods Hole *Photuris,* female, intact, with electrodes on prothoracic ganglion. Stimulus 1 msc./7 v. Temperature 22° C. S = 470 mscs. (59) Maryland *Photinus pyralis,* male, decapitated, electrodes in lantern. Five superimposed responses to 5 mscs./48 v. Temperature 13° C. S = 1 sec. (60) Iowa *Photuris,* male, isolated lantern. Response to two trains of 10 mscs./50 v. at 30 per second. S = 45 secs. (61) *Photinus punctulatus,* male, decapitated, electrodes in prothorax. Electrically excited flashes at 0, 18, 34, and 46 secs. after start of exposure to N_2. S = 760, 520, 680, 1220 mscs. (62) Iowa *Photuris,* male, isolated lantern. Stimulus 1 msc./6 v. at 5 per second for duration of sweep. S = 8 secs. Note that in Figures 63–66 stimulation is indicated by unblanked portion of signal trace. (63) Same as Figure 62 except stimulus 1 msc./5 v. at 15 per second. S = 4.2 secs. (64) Same as Figure 62 except stimulus 1 msc./6 v. at 5 per second. S = 9.5 secs. (65) Same as Figure 62 except stimulus frequency 10 per second. S = 9 secs. (66) Same as Figure 62 except stimulus 1 msc./8 v. at 15 per second. S = 9 secs.

causation, intensity, area of lantern affected, and rates of accretion and decay. Though these are not well understood, they belong in any inventory of the photogenic potentialities of the lantern.

Glowing is the normal mode of luminescence of the larva, and occasionally the larval light organs persist through the pupal stage and are functional in the adult (in the eighth sternite) in addition to the normal adult lanterns in segments 6 and 7. The female of *Photuris* rather typically emits a protracted glow from her adult lantern while in flight, and apparently normal specimens of other species are occasionally seen to emit similar glows. It is not possible to distinguish visually between such normal glows and the continuous luminescence that commonly develops in strong repetitive stimulation and which presumably represents flash fusion (Figs. 23–25, 27, 30, 31, 34, 35, 37 etc.).

In instances where the photomultiplier records flashes superimposed on glow (Figs. 62, 64, 66) it is generally found upon microscopic examination that glowing

regions do not flash, and vice versa. The relations of glow and flash are in fact complex and enigmatical. Glowing may sometimes be suppressed (Fig. 63) or reduced (Fig. 60) by high frequency electrical stimulation, thereafter rising spontaneously though temporarily. It may also apparently be enhanced by repetitive stimulation, after a lag (Figs. 65, 66), with or without flashing.

Sustained glowing is also often engendered by non-physiological conditions, such as excess heat or cold, injection of many materials into the hemocoel, toxic vapors, high or low pO_2, death, etc., and it seems probable that there are several types of "glow," perhaps none of which is analogous to the normal glows of larva and adult, or to the transitory glowing associated with vigorous stimulation.

Microscopic examination of the lantern surface contributes relatively little to our understanding of glows. In most instances, as indeed in flashing, the tissue seems to be uniformly alight, and no fine detail can be made out. The impression is often given that the active tissue is deep in the organ, but there is no histological ground for such a distinction (Buck, 1948) nor is it really possible to distinguish between self-luminosity in surface tissue and secondary glowing due to light diffusing through from a deeper source.

Discussion

Different species of firefly have long been known to differ characteristically in color and intensity of light produced and in flash pattern and duration. The present findings give quantitative expression to the differences in flash duration and form, and show that interspecific distinctions extend to such details of excitation as latency and frequency response limit. No correlations are yet apparent between response characteristics and other species differences such as behavior, body size or lantern structure, except in the instance of the larva, where sluggishness of response is associated with a minute and primitively constructed photogenic organ.

From the electrophysiological standpoint the flash response has so many similarities with conventional neuroeffector excitation systems that direct neural control can scarcely longer be doubted. In regard to the specific analogy with muscle (Chang, 1956), the flash is leisurely and its response latency long compared with a striated muscle twitch, but there is rather good qualitative correspondence between the two systems in regard to strength-duration relations, temperature effects, the effects of changing stimulus voltage, duration and frequency, and the courses of fatigue, adaptation and facilitation. Although we are no nearer to having a concrete picture of how nerve and photocyte are associated, the evidence is strong that the association has many of the properties of a conventional neuroeffector junction.

The remarkable species-specific constancy in normal light emission is coupled with an equally remarkable lability to experimental influences, and it is this capacity for varied response that shows that we are very far from being able to define the basic excitatory event. *A priori,* flash form should be of special interest because of the possibility that it might yield kinetic information. However, in view of the facts that the lantern is estimated to contain of the order of 600,000 photocytes (Buck, 1948) which are controlled in localized groups by peripheral nerve twigs (Hanson, 1961), any hope that the flash mirrors directly the time course of either the basic chemiluminescence or the excitation process is illusory. It has in fact been argued (Buck, 1955) that when light from numerous unresolved foci is de-

tected by an integrating device such as eye or photomultiplier tube, symmetrical, skewed or multiple flashes of any desired form can theoretically be produced merely by varying the gross sequence and spread of excitation. Therefore, although it is possible to give plausible explanations of many of the luminescent phenomena here recorded in terms of conventional interplay between excitation and response mechanisms, it seems preferable to defer most such attempts until conductional, junctional and effector processes can be separated clearly, and the experimental preparation can be limited, if not to single units, at least to constant effector populations.

Nevertheless, the flash is not totally devoid of information. The preferential slowing of the decay phase of the flash at low temperatures (Fig. 46) and in hypoxia (Fig. 61) indicates that the rise and extinction processes are qualitatively different, rather than being merely the two phases of a reversible process. The lability and variability of decay also suggest that it reflects some sort of active control process rather than, for example, dieaway of the ultimate chemiluminescence. The slowing effect of hypoxia on decay of luminescence might be similarly interpreted as interference with a metabolic extinction process. It could also reflect direct oxygen-limitation of the chemiluminescence; however, the virtual absence of fatigue in moderate repetitive stimulation indicates that actual exhaustion of some reactant in light production is unlikely ever to become rate-limiting in the normal over-all response.

Even in the mass flash of the whole lantern there are sometimes clear indications of functional heterogeneity. The flashing of *Photinus consanguineus* is especially interesting in this respect since it normally shows three peaks which have constant latencies (Table I) but independently modulated amplitudes (Fig. 7). The fixed sequence of excitation, shown by both intact and decapitated specimens—but not, significantly, by deganglionated lanterns (Fig. 49)—undoubtedly reflects central nervous programming, while the variable intensities of the three peaks suggest effector populations of variable sizes. Similarly, the early shoulders evoked by intensified stimulation (Figs. 40, 41) probably reflect recruitment of additional groups of effector units with higher threshold and shorter latencies.

A second point of interest about the spontaneous flashing of *P. consanguineus* (Fig. 7) is that whereas each of the three peaks varies continually and markedly in intensity, the total light emitted in each of the last four over-all flashes has an extreme range of variation of only 8% of the mean value, and the first flash is only 25% low. This raises the interesting possibility that the effector units are excited in relays so that total light per over-all flash is kept constant without all photocytes having to participate in each flash. In single-flashing fireflies the maintenance of a uniform flash presents no particular problem, assuming uniform excitation, but the regulation apparently practiced by *P. consanguineus* would appear to require some sort of sensory feedback.

In the pair-shock series shown in Figure 14, both stimuli occur in advance of the response (as long as it is single), so the changes in rise slope and peak position seem reasonably explicable on the basis of conventional facilitation. In the stimulus duration series shown in Figure 49, however, the lantern has no way of knowing, at the time its response begins, how much longer the current will flow—and yet the rise slope progressively flattens and the peak shifts to the right as the stimulus lengthens. Broadening of the flash seems scarcely ascribable to developing inhibi-

tion or to a rescheduling of the firing times of the effector units, because total light increases. It seems, therefore, that although direct current flow may stimulate continuously, producing a response analogous to constant-current contracture of muscle (Fig. 49, last frame; Fig. 50), there may also occur interaction of on and off effects of the pulse. When the pulse is short (Fig. 49, first and second frames) the interaction resembles facilitation and increases the flash height, whereas when start and finish of the pulse are widely separated the effect is seen mainly in a much-prolonged decay phase. Lack of the break effect might also explain why the D.C. threshold is higher than that determined with pulses. In some circumstances, possibly when deganglionation has removed the potential of continuous neural re-excitation, the make and break effects of long pulses occur separately (Fig. 48).

In repetitive spontaneous flashing it is of some interest to inquire how successive flashes are interrelated in magnitude and timing. If we assume a neural pacemaker of not absolute regularity it would be expected that, because of persisting facilitation, shorter interflash intervals would be correlated with more intense succeeding flashes and longer interflash intervals with smaller succeeding flashes. An alternative possibility would be that flash intensity is a function of concentration of substrate available for luminescence—in which case big flashes (using more substrate) should tend to be followed by small, and big flashes should tend to be preceded by longer than average interflash intervals (needed for substrate replenishment). An analysis of the 80 flashes and 79 intervals in the series illustrated in Figure 1 revealed that (a) the flashes following short intervals were 5% more intense than average, (b) the flashes following long intervals were 18% smaller than average, (c) the flashes following big flashes were 7% larger than average, and (d) the flashes following small flashes were 11% smaller than average. We construe these findings to indicate that flash intensity reflects degree of neural facilitation rather than substrate concentration.

The phenomenon or phenomena of induced glowing raises questions of great potential interest, but in view of the paucity of definite information it need only be said that (a) the irreversible, homogeneous, unresponsive, oxygen-sensitive, unchanging glows that are induced by various mechanical or chemical injuries probably represent the uncontrolled luminescing of the substrate stored in the photogenic tissue and are different from either the transitory decay tails of some flashes or the reversible glows associated with high frequency stimulation, (b) the sluggish rate of change of glows, even those responsive to stimulation, cautions against considering them neurally-mediated responses in any usual sense, (c) glows that decay either spontaneously or under stimulation, or glows that augment with stimulation, have some counterparts in flashing behavior; but glows that augment in the absence of stimulation (*e.g.*, Fig. 63) seem to pose an especially interesting problem for the future.

The glow of the firefly larva presumably belongs in a separate category since it is the normal mode of lighting and since it is responsive to quite modest stimulation intensities. In view of the long-known absence from the larval lantern of the tracheal end cells or end organs that form such a characteristic feature of the adult lantern, it seems reasonable to regard the tracheal end cells as being somehow associated with the ability to delimit luminescence sharply. It does not follow, however, that glowing in the adult necessarily means end cell inactivation.

Summary

1. Records are presented of normal spontaneous flashes and of flashes induced by a variety of electrical stimuli at a variety of anatomical sites in several species of lampyrid firefly.

2. The flashes of adult firefly lanterns have long response latencies (25 to 250 mscs. at 25° C. in different species) and durations (100 to 1000 mscs.) and can be repeated many hundred times with only slight fatigue. The response itself shows strength-duration relations and frequency responses (summation, treppe, tetany) which are similar to those of more conventional neuroeffector systems. A striking long-lasting neuroeffector facilitation is also evident.

3. Response latency lengthens with falling temperature, Q_{10} values for the 10°–30° range varying from about 2.4 to 1.4. Extreme temperatures slow the decay phase of luminescence preferentially, as does hypoxia.

4. The flashes of most species differ characteristically in time course, response latency and other electrophysiological properties.

5. The responses of the *Photuris* larva are roughly similar to those of the adult, but slower by a factor of about 10.

6. The time course of light intensity change during flashes induced under various conditions is discussed in possible relation to underlying excitation and effector mechanisms. Long lasting glows are also considered in this context.

Literature Cited

Alexander, Robert S., 1943. Factors controlling firefly luminescence. *J. Cell. Comp. Physiol.*, **22**: 51–71.

Arnold, Carl, 1881. Beiträge zur vergleichenden Physiologie. *Mittheil. Naturforsch. Gesellsch. Bern.*, **1880**: 151–192.

Barber, Herbert Spencer, and Frank A. McDermott, 1951. North American fireflies of the genus *Photuris*. *Smithson. Misc. Coll.*, **117**, No. 1: 1–58.

de Bellesme, Jousset, 1880. Recherches expérimentales sur la phosphorescence du lampyre. *J. Anat. Physiol.*, **16**: 121–169.

Brown, D. E. S., and C. V. King, 1931. The nature of the photogenic response of *Photuris pennsylvanica*. *Physiol. Zoöl.*, **4**: 287–293.

Brücke, E., 1881. Vorlesungen über Physiologie. 3rd ed., **1**: 59–61. Braumüller. Wien.

Buck, John B., 1948. The anatomy and physiology of the light organ in fireflies. *Ann. N. Y. Acad. Sci.*, **49**: 397–482.

Buck, John, 1955. Some reflections on the control of bioluminescence. Pp. 323–332 in: The Luminescence of Biological Systems (Ed. F. H. Johnson), Amer. Assoc. Adv. Sci., Washington.

Carlson, Albert, 1961. Effects of neural activity on the firefly pseudoflash. *Biol. Bull.*, **121**: 265–276.

Case, J. F., and John Buck, 1957. Electrical stimulation of light emission in fireflies. *Biol. Bull.*, **113**: 337.

Case, James, and John Buck, 1958. Excitation of the firefly light organ. *Anat. Rec.*, **132**: 421.

Case, James F., and John Buck, 1959a. Central nervous aspects of firefly excitation. *Biol. Bull.*, **117**: 393.

Case, James F., and John Buck, 1959b. Peripheral aspects of firefly excitation. *Biol. Bull.*, **117**: 407.

Chang, Joseph J., 1956. On the similarity of response of muscle tissue and of lampyrid light organs. *J. Cell. Comp. Physiol.*, **47**: 489–492.

DUBOIS, RAPHAEL, 1886. Contributions à l'étude de la production de la lumière par les êtres vivants. Les Elatérides lumineux. *Bull. Soc. Zool. France*, **11**: 1–275.

FUCHS, SIGMUND, 1891. Einige Versuche an den Leuchtorganen von *Elater noctilucus* L. *Zentralbl. Physiol.*, **5**: 321–325.

GERRETSEN, F. C., 1922. Einige Notizen über das Leuchten des javanishchen Leuchtkäfers (*Luciola vittata* Cast.). *Biol. Zentralbl.*, **42**: 1–9.

GREEN, JOHN WAGENER, 1956. Revision of the nearctic species of *Photinus* (Lampyridae: Coleoptera). *Proc. Calif. Acad. Sci., 4th Ser.*, **28**: 561–613.

HANSON, FRANK E., JR., 1961 (?). Observations on the gross innervation of the firefly light organ. *J. Insect Physiol.* (in press).

HASTINGS, J. WOODLAND, AND JOHN BUCK, 1956. The firefly pseudoflash in relation to photogenic control. *Biol. Bull.*, **111**: 101–113.

HEINEMANN, CARL, 1886. Zur Anatomie und Physiologie der Leuchtorgane mexikanischer Cucujos (*Pyrophorus*). *Arch. mikr. Anat.*, **27**: 296–382.

JOSEPH, GUSTAV, 1854. Beobachtungen über das Leuchten der Johanniskäfer. *Zeitschr. Entom.*, **8**: 1–12.

VON KÖLLIKER, A., 1858. Die Leuchtorganen von *Lampyris*, eine vorläufige Mittheilung. *Verh. phys. med. Ges. Würzburg*, **8**: 217–224.

LUND, E. J., 1911. On the structure, physiology and use of photogenic organs, with special reference to the Lampyridae. *J. Exp. Zool.*, **11**: 415–467.

MACAIRE, J., 1821. Memoire sur la phosphorescence de lampyres. *J. Phys. Chim. Hist. Nat. Arts*, **93**: 46–56. Repeated in *Ann. Chim. Phys.*, **17**: 151–167.

MACARTNEY, J., 1810. Observations on luminous animals. *Phil. Trans. Roy. Soc.*, **100**: 258–293.

OWSJANNIKOW, PH., 1868. Ein Beitrag zur Kenntniss der Leuchtorgane von *Lampyris noctiluca*. *Mem. Acad. Imp. Sci. St. Petersburg, Ser. 7*, **11**: No. 17: 1–12.

PERKINS, MICHAEL, 1931. Light of glow-worms. *Nature*, **128**: 905.

SNELL, PETER A., 1932. The control of luminescence in the male lampyrid firefly, *Photuris pennsylvanica*, with special reference to the effect of oxygen tension on flashing. *J. Cell. Comp. Physiol.*, **1**: 37–51.

STEINACH, E., 1908. Die Summation einzeln unwirksamer Reize als allgemeine Lebenserscheinung. Vergleichend-physiologische Untersuchungen. *Arch. ges. Physiol.*, **125**: 239–346 (see pp. 284–289).

TODD, T. J., 1826. An inquiry into the nature of the luminous power of some of the Lampyrides, etc. *Quart. J. Lit. Sci. Arts*, **21**: 241–251.

VERWORN, MAX, 1892. Ein automatisches Centrum für die lichtproduction bei *Luciola italica* L. *Zentralbl. Physiol.*, **6**: 69–74.

VARIATIONS IN THE DORSAL PATTERN OF CYATHURA POLITA (STIMPSON) FROM ESTUARIES ALONG THE COASTS OF EASTERN UNITED STATES AND THE GULF OF MEXICO[1]

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It has been established that the isopod which has been collected in coastal estuaries and tidal marshes of the United States from Louisiana to Maine belongs to a single species, *Cyathura polita* (Stimpson) (Miller and Burbanck, 1961). Any species having such an extensive distribution might be expected to show some morphological variation, and if such variation were inherited, geographical races within the species might be recognizable. Differences in pattern and coloration are genetically controlled in certain crustaceans. In *Tisbe reticulata* (Bocquet, 1951; Battaglia, 1958), there is inheritance both of color and of the distribution of the violet, rose, and light brown pigments within the sectors of a dorsal, hypodermal network. *Sphaeroma serratum* (Bocquet, Lévi and Teissier, 1951) also has distinct colored forms which can be classified and for which the mode of inheritance can be determined. The presence or absence of chromatophores form inheritable patterns in *Jaera marina* (Bocquet, 1953, 1954) and *Cambarellus shulfeldtii* (Volpe and Penn, 1957). The forms of *Jaera* were distinguished chiefly on the basis of colored and uncolored bands which corresponded to the segments of the body. There was also a pattern of small white dots, usually elliptical in shape, the "impressions musculaires," which was evident on pigmented segments, but this pattern remained essentially the same in all phenotypes recognized. *Cyathura polita* has an intricate and distinct dorsal pattern formed by light areas from which chromatophores are absent alternating with areas filled by dark chromatophores (Fig. 1). These light areas appear to correspond to the less extensive "impressions musculaires" of *Jaera marina* and to be areas of attachment of muscles.

Casual examination of cyathurans shows a marked similarity of pattern design in all the individuals. During the course of extensive studies of *Cyathura polita* (Burbanck, 1959; Miller and Burbanck, 1961; Burbanck, 1961a; Burbanck, 1961b), it was noted that the pattern was most clearly defined on the head and on the first two thoracic segments. When it was noticed that the pattern of animals collected from the Ashepoo River, South Carolina, looked "different," the arrangement of chromatophores in the anterior segments was compared with that of cyathurans collected in other areas, and it was decided to make a comparative study of the central portion of the pattern of the first thoracic segment. This configuration of chromatophores was chosen because it showed two types of variation which could be used in a comparative study, *viz.*, a variable number of uncolored areas and differences in their orientation.

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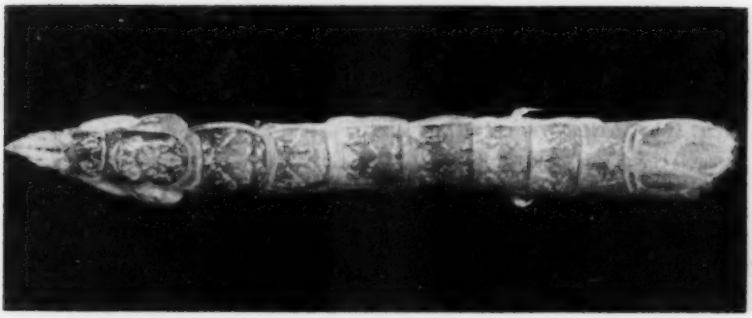


FIGURE 1. Female *Cyathura polita* from Silver Glen Springs, Florida, 19 mm. in length. Photograph courtesy of Dr. E. L. Hunt.

MATERIALS AND METHODS

To determine if there was a recognizable pattern difference between geographically separated populations, animals from two areas from which large samples had been obtained were compared. Ninety-five animals collected in the Silver Glen-Juniper Springs area of Lake George, Florida, in 1959 and 1960 were compared with a total of 117 animals collected from the Pocasset River, Massachusetts, in May and June of 1960. In addition to these large collections, specimens from the entire range of *Cyathura polita* were examined. These included the animals collected by W. D. Burbanck and reported on in 1959, and more recent collections from the Gulf of Mexico, the Hudson River, the coasts of Connecticut and Rhode Island, and Cape Cod, Massachusetts.

Cyathurans were preserved in 70% alcohol and glycerine; after five years in this preservative, the chromatophore pattern is still distinct. Photographs, camera lucida drawings, sketches, and diagrams were made of the central part of the pattern of the first thoracic segment. This central unit, roughly bilaterally symmetrical as is the whole dorsal pattern, was usually composed of 5 or 6 uncolored areas or "parts"—two larger parts each with a vertical portion (V in Fig. 2) and a divergent arm (A in Fig. 2) and 2–4 smaller, basal parts (B in Fig. 2) arranged in various ways with respect to the larger parts. When analyzing large numbers of animals, it was convenient to use diagrams of the pattern. Thus, Figure 2 is a diagrammatic representation of the pattern photographed in Figure 3.

FIGURE 2. Diagram of central part of pattern on first thoracic segment of a cyathuran from Pocasset River, Massachusetts. V = vertical portion of large part; A = divergent arm; B = basal part.

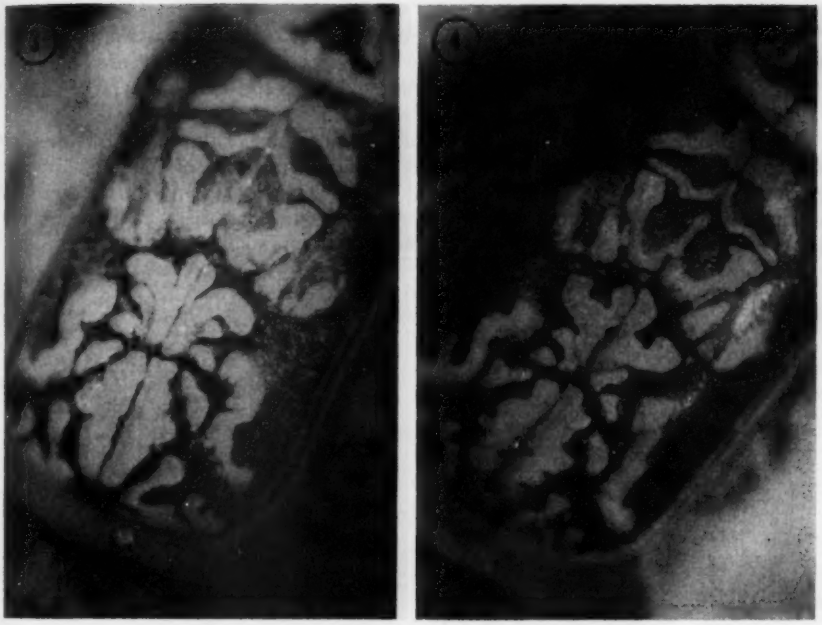


FIGURE 3. First thoracic segment of male collected at Pocasset River, Massachusetts, May 15, 1960.
FIGURE 4. First thoracic segment of a female collected at Silver Glen Springs, Jan. 2, 1960.

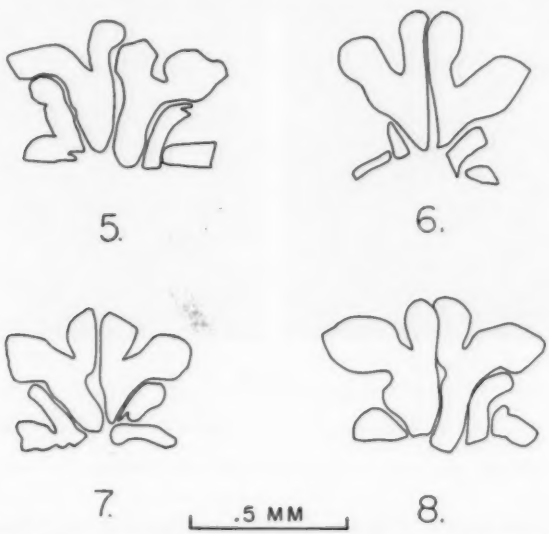
Juveniles about 8 mm. in length and larger showed as clear and characteristic a pattern as did adult animals. The dorsal patterns of very small animals had an "unformed" appearance and were not included in this study.

OBSERVATIONS AND RESULTS

An analysis of the patterns of the Lake George, Florida, and the Pocasset River, Massachusetts, animals revealed that there was a basic difference in the orientation of the parts of the pattern studied. In the southern animals, the arms of the pattern are oriented upward (Figs. 4 and 6), and in the northern animals one or both of the arms tend to angle horizontally or downward (Figs. 3 and 5). This difference is not absolute. Of the 95 Lake George animals examined, 82 animals showed the upward orientation; 11 were of the northern type, two with both arms horizontal and 9 with one arm oriented upward and the other horizontally; and in two animals the pattern was abnormal. A pattern was considered abnormal if it was fragmented into 10 or more parts or if it was fused and distorted from the usual shape. If there was abnormality in the part under analysis for this study, there was usually distor-

tion of the pattern of the whole animal. Of the 116 animals from Pocasset River, 93 showed the northern type of orientation, 65 with both arms horizontal and 28 with only one arm horizontal; 22 were of the southern type; and 2 were abnormal. Thus, 86% of the animals from Lake George exhibited the southern type of pattern and 79% of the Pocasset River animals were of the northern type.

The number of component parts of this central figure of the pattern of the first thoracic segment varies from 8 to a fusion into one single mass. The usual range is from 4–6 parts with the mode for the northern animals at 5 and for the southern animals at 6. This difference is not so striking, however, if the average number of parts is compared—4.93 for Pocasset animals, 5.40 for Lake George animals.



FIGURES 5–8. Camera lucida drawings of central part of pattern of first thoracic segments. Figure 5: Female from Pocasset River, Massachusetts, June 12, 1960. Figure 6: Female from Silver Glen Springs, Florida, Jan. 2, 1960. Figure 7: Female from upper reaches of Bass River, Massachusetts, Oct. 26, 1960. Figure 8: Male from Stony Brook, Massachusetts, Oct. 12, 1960.

In addition to the number of parts, the arrangement of the parts tends to differ with the geographical origin of the animals. In the Pocasset River animals, the part of the pattern studied tends to be "squared off" at the base; the two large parts extend down to a hypothetical base line and the small parts on either side rest on this "base line." (See Figs. 3, 5, 7, 8.) If both arms are horizontal with the upper edges roughly parallel with this base line, the whole effect is one of rectangularity. The pattern of Lake George animals tends to be elongated rather than squared. As already noted, the arms reach upward, often with a widely flared V between the arm and the central vertical part (Fig. 6). Instead of the small pieces being arranged at the base beside the vertical part, the lower end of the vertical part may be truncated and the small pieces arranged as a downward but interrupted continua-

tion of the vertical segment (Fig. 6), flaring out to the side but not lined up and squared off as in many of the Pocasset River animals. This difference in the arrangement of the parts of the pattern cannot be analyzed as precisely as the orientation of the arms or the number of the fragments but is recognizable in a majority of the specimens.

Two additional sizeable collections of *Cyathura polita* were made in the fall of 1960 in connection with other aspects of cyathuran research and were available for pattern analysis. The patterns of 51 animals collected by dredging the upper three-fourths of the Pocasset River substantiated the characterization of the Pocasset population as to the orientation of the arms and the squaring off of the basal parts, but patterns containing 5 and 6 parts occurred in about equal numbers. Forty-six cyathurans collected late in October in one sample from Stepping Stone Creek, north of Chatham on Cape Cod, were almost entirely northern as to orientation and arrangement of the pattern but again the number of parts did not exhibit a pronounced mode of five as did the spring collection of Pocasset animals.

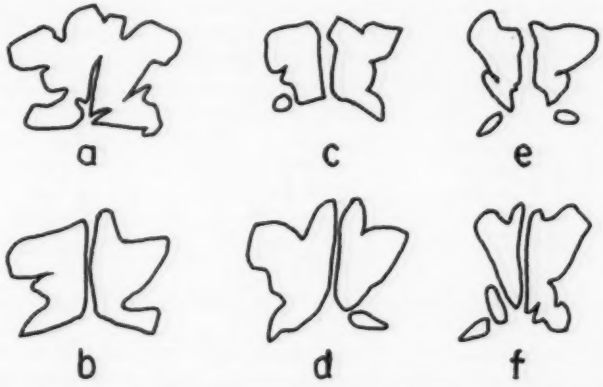


FIGURE 9. Sketches of patterns from animals from the extremes of the known range of *Cyathura polita*. a: Cider Hill Creek, Maine. b: Lake Pontchartrain, Louisiana. c and d: St. Louis Bay, Mississippi. e and f: Perdido Bay, Alabama.

A survey of animals collected along the Atlantic Coast of the United States corroborated the division of *Cyathura polita* into two groups on the basis of dorsal pattern. Although the samples examined were relatively small (1–15 animals with the exception of larger special collections from Cape Cod, Mass., and 26 animals from Beacon, N. Y.), animals from the Hudson River northward were chiefly of the northern type, and animals from New Jersey to Florida were predominantly of the southern type.

There was a tendency for animals at the extremes of the known geographical range of *C. polita* to show a modification of the basic design of the pattern. At the northern extreme in Maine the general rectangular, northern type of outline remained the same, but there was frequently a fusion of parts of the pattern even across the midline. In samples of only ten animals, there would be one animal in

which the part of the pattern being studied was fused into one piece (Fig. 9a). At the southwestern extreme along the Gulf of Mexico in Alabama, Mississippi, and Louisiana, there was a similar modification in that the number of parts was reduced and the upward orientation of the arms was less pronounced than in the Lake George animals. The pattern of animals from Lake Pontchartrain, Louisiana (Fig. 9b), was usually made up of two halves which were not further subdivided into parts and which were more suggestive in outline of the squared-off northern pattern than of the southern pattern as typified by the Lake George animals. Eastward from Louisiana, animals from Mississippi and Alabama showed patterns intermediate between the Louisiana and Lake George, Florida, types. The Mississippi animals were very similar to the Louisiana ones but half of them had the basal part of one side of the pattern separate from the main part (Figs. 9c, 9d). The Alabama animals were intermediate between the Florida type and the Mississippi and Louisiana patterns; in Figure 9e, the arms are suggestive of the northern and Louisiana type but the orientation of the parts at the base is as in Florida animals. Other Alabama animals were as in Figure 9f, where the outline is that of the southern type and the number of parts is less than is usual for the southern type of pattern but more than for Louisiana and Mississippi animals. The maximum number of parts (except for one abnormal pattern which had 6) for the ten Alabama animals was four, shown by five animals.

FIGURE 10. Sketches of patterns of three animals from Ashepoo River, South Carolina.

It was the observation of a fusion of parts similar to that seen at the extremes of the range of *Cyathura polita* which was noted in the animals from the Ashepoo River, South Carolina, and which initiated this study (Fig. 10). Comparison of the pattern of animals from the Ashepoo River with Maine, Pocasset River, Lake George, and Gulf of Mexico animals reveals that the orientation of the arms is that of the southern type and the fusion of the parts is similar to that of the Gulf and Maine animals with the additional feature of a high proportion of animals with the pattern fused across the midline.

Late in 1960, the opportunity arose to examine about 500 cyathurans collected in 34 estuaries of Cape Cod, Massachusetts, in connection with a study of the distribution of *Cyathura* in the Cape Cod area (Burbanck, 1961b). There were usually about 10 animals per sample, but in two instances there were about 50 and in three other collections there were 29–30 animals each. The northern type of pattern was present in 93% of these animals, and the average number of parts was 5.0. The general aspect of the majority of the patterns was rectangular as may be considered characteristic of the northern type of pattern, but there was a variation recognizable in enough instances to suggest that it might be a Cape Cod variant, a modified northern type. Particularly in the collections from two relatively isolated

populations, the upper reaches of Bass River (29 animals) and Stony Brook (11 animals), there were animals with a pattern different from the typical Pocasset River animals (Figs. 7, 8). Of the 29 animals collected from the run-off from Mill Pond in the upper reaches of Bass River, only in the patterns of 9 animals were both arms directed horizontally. In 16 animals one arm was horizontal with roughly parallel sides but the other was neither directed upward as in the southern type nor horizontally or downward with parallel sides as in the typically northern pattern. Not only was this an unusually high percentage of animals with a half-and-half orientation of the arms, but the one northern type arm was usually not as extended as in half-and-half animals from Pocasset River (compare Figures 5 and 7).

Collections from nearby Lake Tashmoo and Lagoon Pond on Martha's Vineyard Island, Massachusetts, made in 1959 and 1960 were also of the northern type. The patterns of those from Lagoon Pond resembled those from the extremes of the range in that the number of parts was reduced, and in one collection of 12 animals, the pattern was fused across the midline in 7 of them.

Whenever collections were made from the same locality over a period of several years, the patterns did not vary appreciably. Animals collected from the Silver Glen Spring area of Lake George, Florida, in 1956, 1959, and 1960 all exhibit patterns which are predominantly of the southern type with very little variation. Collections from Pocasset River have been made yearly since 1955 and the pattern of all animals examined has been consistent within a normal range of variation regardless of the year of collection. Even some of the peculiarities of pattern observed in certain Cape Cod animals collected in 1960 were present in earlier collections from the same places.

Discussion

The persistence over a period of years of a recognizable difference in pattern suggests that the pattern design may be an inherited character. With such a wide range of climatic conditions from Louisiana, around Florida, and north to Maine, it was anticipated that the chromatophore pattern might vary and that there might be populations which could be considered distinct races within the species. Specimens of *C. polita* from its whole known geographical range have been examined and found to be similar enough morphologically to be considered one species (Miller and Burbanck, 1961). The variability of the chromatophore pattern, however, and the correlation of two distinct pattern types with geographical distribution, raises the question whether there may be ecotypes within the species *Cyathura polita*.

Three of the four levels of variability postulated by Clausen and Hiesey (1958) in their analysis of ecological races in the *Potentilla glandulosa* complex are recognizable in *Cyathura polita*. There is variation of pattern within the local populations which is the first level of variability according to Clausen and Hiesey. The second level is that of varying, isolated, local populations, within the same climatic range and under similar edaphic conditions. The populations of *Cyathura* along the Gulf coast were distinct as to pattern design but living under similar environmental conditions. The variation noted on Cape Cod may also be at this level as may be the population from Lagoon Pond, Martha's Vineyard Island. The

third level of variability is defined as that distinguishing populations in climatically or edaphically different habitats. In the case of *Potentilla glandulosa* they were ecotypes with physiological differences sometimes accompanied by morphological differences. The existence of a northern type pattern and a southern type in *C. polita* is suggestive of this level of variability. Further investigation needs to be made of other morphological variations, such as the anatomy of the appendix masculinum (Miller and Burbanck, 1961) and chromosome numbers, and physiological studies will be needed of the tolerances of the animal before the hypothesis of the existence of ecotypes within the species can be verified. The fourth level of variation distinguished in *Potentilla* is the establishment of morpologically and physiologically distinct taxonomic subspecies. On the basis of present knowledge, there is no evidence that *Cyathura polita* has reached this level of variability and differentiation.

Until more is known about the breeding habits of *C. polita* and animals can be successfully kept under laboratory conditions for longer periods of time than at present, a genetic analysis of the inheritance of pattern types cannot be made. The present picture is suggestive of a wide-ranging, interbreeding (?) population within which have evolved two types of dorsal pattern under two different types of climate, northern and southern. The pattern types may form a graded series or cline, but to confirm this it would be necessary to examine larger collections of animals from the populations between Cape Cod and Lake George as well as from the extremes of the range. There is evidence of specialization of the pattern at the extremes of the range and in isolated populations of both the northern and southern type animals. The regional peculiarity of pattern noted in the cyathurans from the Ashepoo River, South Carolina, was an example of the development of variation in an isolated population, a population which disappeared after a hurricane struck this area in the fall of 1959 (Burbanck, 1961).

LITERATURE CITED

Battaglia, B., 1958. Balanced polymorphism in *Tisbe reticulata*, a marine copepod. *Evolution*, **12**: 358–364.

Bocquet, C., 1951. Recherches sur *Tisbe* (= *Idyaea*) *reticulata*, n. sp. *Arch. Zool. Exp. et Gén.*, **87**: 337–416.

Bocquet, C., 1953. Recherches sur le polymorphisme natural des *Jaera marina* (Fabr.) (Isopodes Asellotes). *Arch. Zool. Exp. et. Gén.*, **90**: 187–450.

Bocquet, C., 1954. Evolution of a superspecies of marine isopods. *Syst. Zool.*, **3**: 148–162.

Bocquet, C., C. Lévi and G. Teissier, 1951. Recherches sur le polychromatisme de *Sphaeroma serratum* (F). *Arch. Zool. Exp. et Gén.*, **87**: 245–297.

Burbanck, W. D., 1959. The distribution of the estuarine isopod, *Cyathura* sp., along the eastern coast of the United States. *Ecology*, **40**: 507–511.

Burbanck, W. D., 1961a. The disappearance of *Cyathura polita* (Stimpson) from the Ashepoo River, South Carolina, after hurricane "Gracie" in 1959. Ecology, **42**: 606–607.

Burbanck, W. D., 1961b. The distribution and ecology of *Cyathura polita* (Stimpson) in tidal marshes of Cape Cod, Massachusetts, and its vicinity. *ASB Bull.*, **8**: 29–30.

Clausen, J., and W. M. Hiesey, 1958. Experimental studies on the nature of species. IV. Genetic structure of ecological races. *Carnegie Inst. Wash. Pub.* **615**. Washington, D. C. iv + 312 pp.

Miller, M. A., and W. D. Burbanck, 1961. Systematics and distribution of an estuarine isopod crustacean, *Cyathura polita* (Stimpson, 1855), new comb., from the Gulf and Atlantic seaboard of the United States. *Biol. Bull.*, **120**: 62–84.

Volpe, E. P., and G. H. Penn, 1957. Dimorphism of chromatophore patterns in the dwarf crawfish. *J. Heredity*, **48**: 91–96.

EFFECTS OF NEURAL ACTIVITY ON THE FIREFLY PSEUDOFLASH[1,2]

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The pseudoflash of the adult firefly, first recorded by Snell in 1932, is produced by subjecting the animal to a sudden increase in oxygen concentration after anoxia. A dull, hypoxic glow develops during anoxia and spreads over the entire organ, gains in intensity, then slowly declines to extinction. If air is readmitted at any time during the hypoxic glow, there occurs a brilliant pseudoflash of one second or longer. According to Snell and later, Alexander (1943), the pseudoflash reflects the unimpeded entry of oxygen into the luminous tissue through supposedly valvular tracheal end cells (Dahlgren, 1917), the valves being temporarily inactivated by hypoxia. This theory implies that firefly luminescence is normally oxygen-limited and that the pseudoflash is independent of neural activity.

McElroy and his associates (summarized in McElroy and Hastings, 1955) suggest the possibility of luminescence control by chemical reactions not directly involving oxygen, so that the light organ may be oxygenated at all times. Further, these workers have proposed a flash-generating mechanism by which the nerve impulse brings about release of pyrophosphate which in turn stimulates the light-yielding reaction.

Hastings and Buck (1956) concluded from experiments on decapitated adults, isolated light organ segments and excised photogenic organs, that nerve impulses originating in the central nervous system played no role in the photogenic response to hypoxia since in all these preparations an hypoxic glow developed in low oxygen concentrations and a pseudoflash resulted with readmission of air. They further noted, as had others previously, that pseudoflash and hypoxic glow involved only one or a portion of one segment in some cases, that some animals failed to give either response, and that considerable variation in both responses occurred even within one individual.

The variations of hypoxic response of individual fireflies, ranging from no response during quiescent periods to complete, two-segment responses in actively flashing animals, indicate that factors besides hypoxia play a role in pseudoflash production. A number of observations, previously reported in abstract form (Carlson, 1959), strongly implicate the nervous system. The purpose of this

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[3] The research reported here was completed in the Department of Zoology, State University of Iowa, and at The Marine Biological Laboratory, Woods Hole, Massachusetts.

report is to present in detail these observations concerning the role of the innervation in pseudoflash and hypoxic glow.

Materials and Methods

Seventy-four adults of two firefly species, *Photinus pyralis* Linn. and *Photuris* sp., were the subjects of this study. Fireflies were exposed to various concentrations of oxygen in nitrogen and to pure nitrogen. The experimental animal was secured ventral side up on a narrow glass spatula provided with platinum stimulating electrodes. The spatula was placed immediately next to the gas inlet in a glass tube one foot long and one centimeter in diameter. A photomultiplier tube (RCA 931-A) and dissecting microscope were positioned above the gas chamber and both were shielded by black cloth from stray light. Gases entered the chamber through a Y-tube provided with stopcocks arranged to permit rapid alternation of two gas mixtures. Gases were prepared from commercial compressed nitrogen and oxygen metered through two-stage reduction valves and calibrated Fischer-Porter flow meters. Mixture composition was checked by gas analysis with a Scholander 0.5-cc. analyser.

In preparation for a pseudoflash one valve was rotated 180° to shunt off the oxygen and admit either pure nitrogen or a nitrogen-oxygen mixture containing

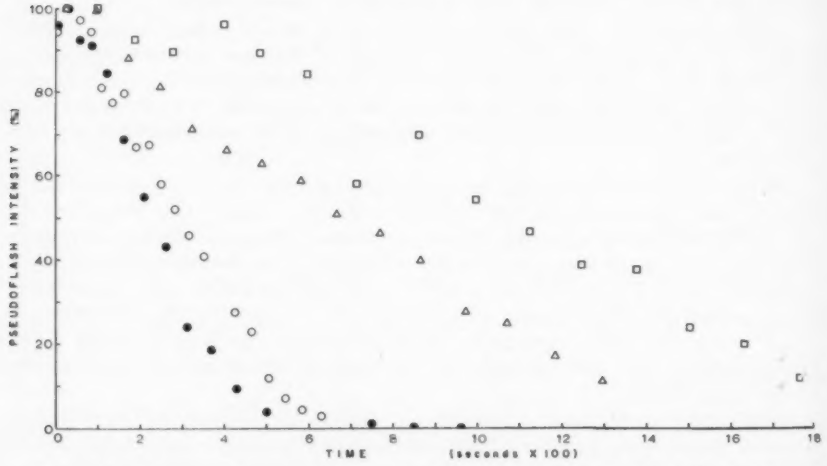

Figure 1. Decrease of pseudoflash intensity in successive pseudoflashes induced at hypoxic glow maximum with recovery periods of constant duration in adult males, *Photinus pyralis*. Ordinate: Pseudoflash intensity in per cent of most intense pseudoflash in each series.

In this and following graphs open circles denote successive pseudoflashes with a constant air recovery period of 10 seconds. Closed circles denote a second run with the same animal immediately after inducing the animal to flash and with a constant air recovery duration of 11 seconds. Squares denote successive pseudoflashes in another animal with a constant air recovery duration of 60 seconds. Triangles denote successive pseudoflashes in a third animal with a constant air recovery duration of 30 seconds. Pseudoflashes in all cases were induced with oxygen concentration of 17.25%. Oxygen concentration during hypoxia was 0.5%.

less than 2.5% oxygen. Rotation of this stopcock also opened a signal circuit. At an appropriate time the same valve was then rotated back 180° which allowed a higher concentration of oxygen to reach the animal suddenly and also closed the signal circuit. The pseudoflash was detected by the photomultiplier, and its output was led to one or both channels of an Offner Dynograph.

Nerve action potentials in the adult light organ during pseudoflash generation were recorded with the animal in a chamber with inlet and outlet tubes and covered with a transparent plastic sheet. Uninsulated platinum recording electrodes were placed on the light organ surface after cuticle removal. Action potentials were led through an AC amplifier to one beam of an oscilloscope. The other beam recorded the photomultiplier output.

Results

1. *Induction of pseudoflashes in Photinus adults*

In actively flashing animals hypoxia initially produces a dark period of five seconds or less, followed by an hypoxic glow, which begins suddenly as a dull glow over the entire lantern, then gradually and uniformly brightens. Pseudoflashes induced after the hypoxic glow develops are of high intensity. In quiescent, non-flashing adults either no hypoxic glow and pseudoflash can be induced even with prolonged hypoxia, or a very dull, spotty, hypoxic glow develops after a dark period lasting for minutes. The subsequent pseudoflash is greatly reduced as compared with those induced in individuals with bright hypoxic glows. If the quiescent animal is first stimulated to flash, its capacity for developing a bright hypoxic glow and pseudoflash is greatly enhanced.

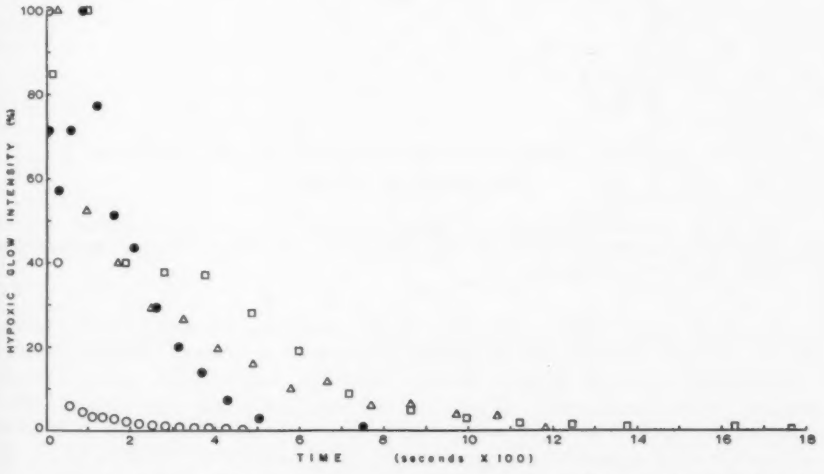

FIGURE 2. Decrease of hypoxic glow maximum intensity with successive pseudoflashes and with recovery periods of constant duration in adult males, *Photinus pyralis*. Ordinate: Hypoxic glow intensity in per cent of most intense hypoxic glow in each series.

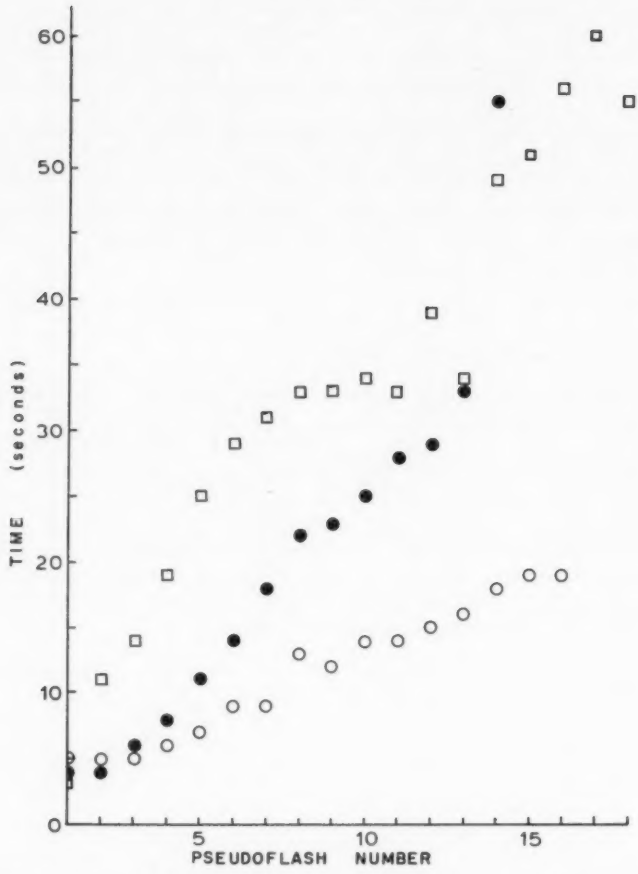

FIGURE 3. Increase in duration of hypoxia prior to the onset of the hypoxic glow in successive pseudoflashes in adult males, *Photinus pyralis*. Ordinate: Hypoxic duration prior to hypoxic glow onset in seconds.

In spontaneously flashing *Photinus* adults a large number of successive pseudoflashes of high intensity can be induced. However, quiescent animals exhibit a characteristic series of changes during successive pseudoflashes which strongly suggest neural involvement. If such animals are first induced to flash spontaneously, then exposed to a given hypoxic oxygen concentration during attainment of hypoxic glow maximum, alternating with fixed periods of exposure to air, the pseudoflashes produced go through the following progressive changes:

(1) Pseudoflash intensity slowly declines (Fig. 1). (2) Hypoxic glow intensity rapidly declines initially, then declines at a slower rate (Fig. 2). (3) The

time required from onset of hypoxia to onset of the hypoxic glow increases (Fig. 3). (4) The time from onset of hypoxia to the attainment of maximal hypoxic glow increases (Fig. 4). (5) Animals that have undergone these changes can be restored to their original condition of intense hypoxic glow and pseudoflash by stimulating mechanically or electrically until they begin to flash spontaneously (Figures 1, 3 and 4; open and closed circles showing two successive runs with the same animal). The rate at which these progressive changes occur is highly variable not only among different individuals but also within the same individual at different times. The graphs indicate the range of effects observed in a total of 30 runs on 13 individuals studied.

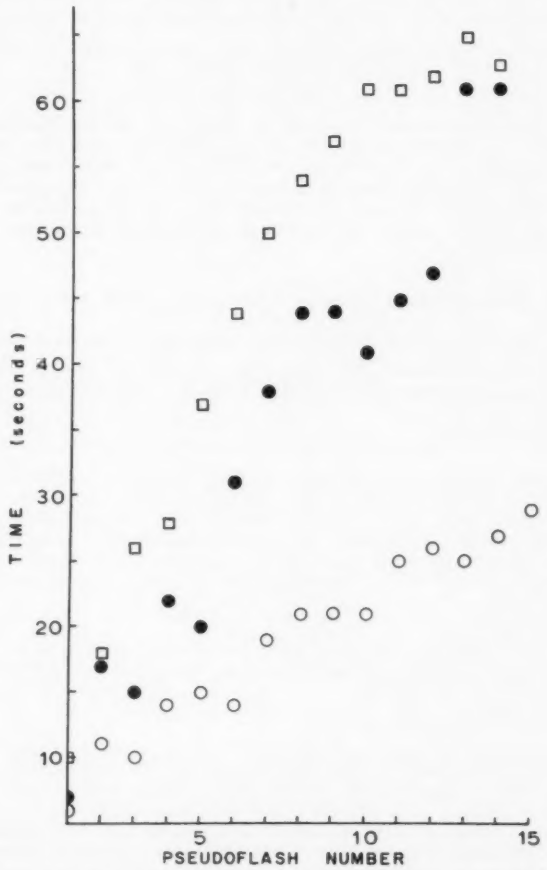

FIGURE 4. Increase in duration of hypoxia prior to attainment of maximum hypoxic glow intensity with successive pseudoflashes in adult males, *Photinus pyralis*. Ordinate: Hypoxic duration prior to hypoxic glow maximum intensity in seconds.

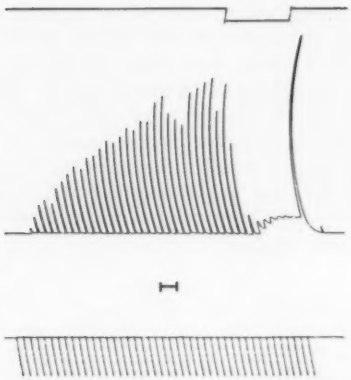

FIGURE 5. Electrically excited neural flashes in air and during hypoxia, followed by a single pseudoflash in an adult male, *Photinus pyralis*. Time axis reads from left to right. Top trace: up, 17.9% oxygen; down, .065% oxygen. Middle trace: light intensity. Bottom trace: stimulus signal. One stimulating electrode in anterior abdomen and other dorsal to light organ through anus. Stimulation: 8 volts, 50-msec. duration, frequency 0.5 per second. Heavy horizontal line represents 5 seconds on this and all subsequent figures except where otherwise noted.

2. *Effects of electrical stimulation on hypoxic glow and pseudoflash*

Electrical stimulation of a firefly in air normally produces a facilitating series of flashes. When stimulation continues into the hypoxic period, flashes diminish and a high intensity hypoxic glow appears (Fig. 5). A pseudoflash can be induced

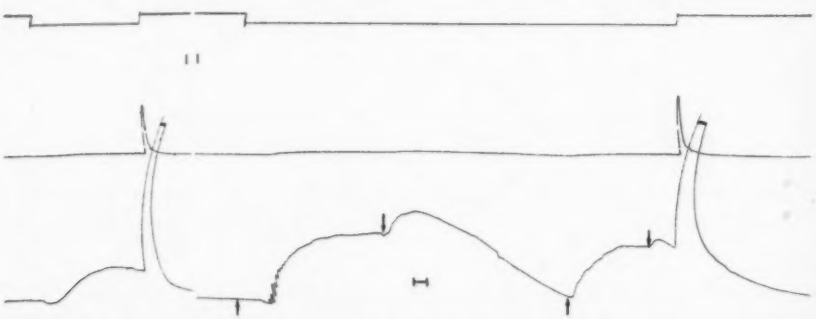

FIGURE 6. Effect of electrical stimulation during hypoxia upon hypoxic glow in an adult male, *Photinus pyralis*. Top trace: up, 17.25% oxygen; down, .05% oxygen. The middle and bottom traces both indicate light intensity, the bottom trace at 10 times the amplification of the middle trace. Stimulating electrode pair in abdomen dorsal to light organ. Stimulation: 5 volts, 50-msec. duration, frequency 1 per second. Short parallel lines indicate a break in the record. In this figure and subsequent figures where used, arrows pointing up indicate stimulus onset and arrows pointing down indicate stimulus cessation. The figure shows first an anoxic sequence without electrical excitation followed by two stimulus trains during hypoxia. In last two episodes glow rose temporarily after stimulation ceased.

as soon as electrically excited flashes undergo an increase in duration, which appears to be characteristic of the hypoxic state. Electrical stimulation during hypoxia increases the rate of hypoxic glow rise and intensity of glow attained (Fig. 6). It will also be noted that when stimulation ceases a rapid brightening usually occurs followed by a decline, an effect which is typically observed. The hypoxic glow was maintained in one animal for 20 minutes by periodic stimulation in 0.6% oxygen; without stimulation it might be expected to persist less than two minutes.

Although prolonged stimulation during hypoxia favors a pseudoflash of increased duration, stimulation, when continued during readmission of air, superimposes no discrete flashes on the pseudoflash rise. However, this may occur during pseudoflash decay as illustrated in Figure 7. Electrical stimulation commonly augments the hypoxic glow by recruiting new, discrete luminescing areas

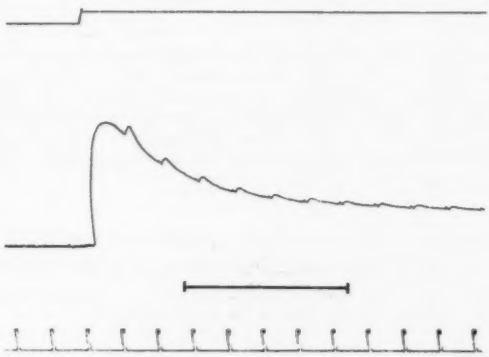

FIGURE 7. Electrical stimulation during readmission of 17.75% oxygen and consequent pseudoflash in an adult male, *Photinus pyralis*. Top trace: up, 17.75% oxygen; down, .05% oxygen. Middle trace: light intensity. Bottom trace: stimulus signal. Stimulating electrode pair placed in sides of abdomen dorsal to light organ. Stimulation: 50 volts, 40-msec. duration, frequency 1 per second. Tiny marks on middle trace most apparent during hypoxia are stimulus artifacts via ground circuit, not light emission.

of the lantern in serial fashion, one or more areas per pulse. The effect is illustrated in Figure 8; each pulse initiates luminescence which covers one additional area completely.

3. *Effects of an anticholinesterase and of denervation on hypoxic glow and pseudoflash*

In order to examine further the role of possible neural activity on the pseudoflash, the effects of injection of an anticholinesterase and of nerve transection on the hypoxic glow and pseudoflash were studied. Eserine, 10^{-4} *M*, in Roeder's saline (Roeder, 1953), injected into the body cavity of the decapitated adult, after removing the tip of the abdomen to permit flow of the solution through the body,

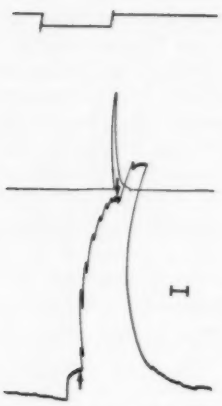

FIGURE 8. Effects of electrical stimulation during rising phase of hypoxic glow in an adult male, *Photinus pyralis*. With each shock hypoxic glow augmented as new areas begin to glow and remain glowing. Same effect can be noted in Figure 6, after first arrow. Top trace: up, 17.25% oxygen; down, .05% oxygen. Middle and bottom traces: light intensity, amplification of bottom trace being 1000 times middle trace. Stimulating electrode pair on right and left sides of seventh abdominal segment dorsal to light organ. Stimulation: 10 volts, 50-msec. duration, frequency 1 per second.

produces nearly continual high intensity scintillation and flashing in air (Case and Buck, 1959). Under these conditions the rate of rise and maximal intensity of hypoxic glow are increased greatly. Pseudoflashes of normal characteristics can be induced after as little as 5 seconds of hypoxia (Fig. 9).

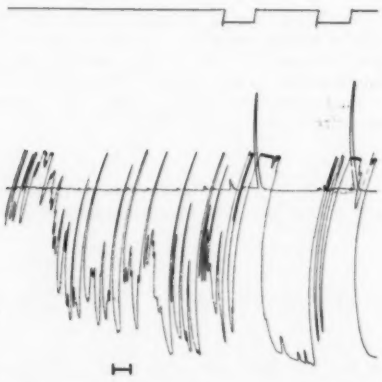

FIGURE 9. Effect of perfusion of 10^{-4} *M* eserine through a decapitated adult male, *Photinus pyralis*. Note the continual scintillation and rapid onset of hypoxic glow. Top trace: up, 17.25% oxygen; down, .05% oxygen. Middle and bottom traces: light intensity, amplification of bottom trace being 200 times middle trace.

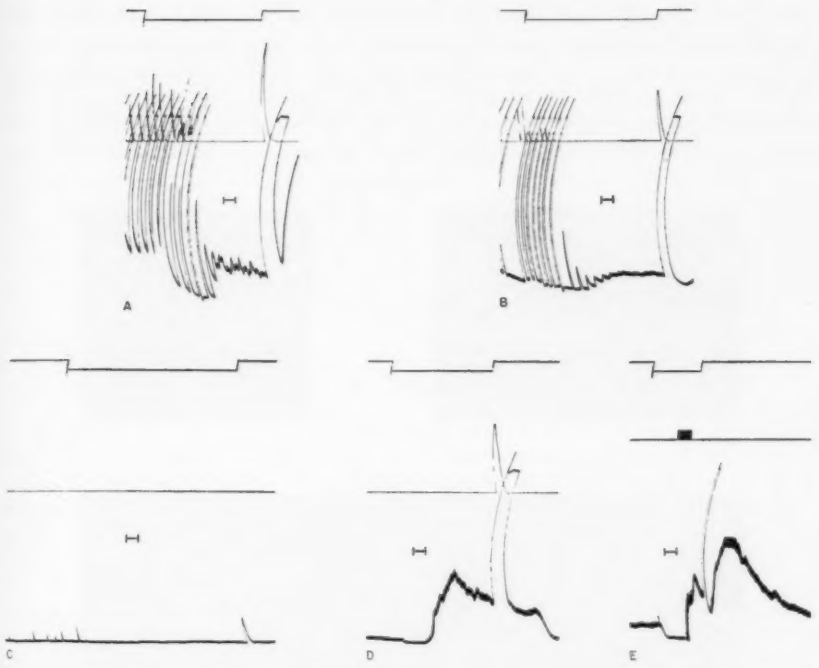

FIGURE 10. Effect of transection of the ventral nerve cord between the sixth and seventh abdominal segments of a female, *Photuris sp.* Except where noted otherwise the following conditions were maintained. Top trace: up, 21% oxygen; down, .05% oxygen. Middle and bottom traces: light intensity except where otherwise noted, amplification of bottom trace being 500 times middle trace. (A) Normal pseudoflash, both sixth and seventh abdominal segments give a total flash. Spontaneous flashing is seen in this figure, as well as in B, during onset of hypoxia. (B) Normal pseudoflash, sixth segment masked. Note the reduction in intensity of the pseudoflash since only the seventh segment is being recorded. (C) Inability to induce pseudoflash in segment 7, 8 minutes after transection, sixth segment masked. Top trace: up, 21.4% oxygen; down, 0.4% oxygen. Light leak from sixth segment appears as small flashes on left, bottom trace. (D) Pseudoflash 20 minutes after transection, seventh segment masked, showing response in sixth segment still normal. Amplification of light signal in bottom trace, 1000 times middle trace. (E) Pseudoflash 45 minutes after transection with stimulation, sixth segment masked. Stimulating electrode pair inserted laterally into the seventh segment dorsal to the light organ. Middle trace: stimulus signal. Bottom trace: light intensity with same amplification as before. Stimulation: 15 volts, 20-msec. duration, frequency 20 per second.

In some instances, transection of nerves supplying the light organ of abdominal segment 7, at the level of the intersegmental membrane between segments 6 and 7, abolishes spontaneous flashing in the seventh segment and leaves unaffected the light organ of segment 6. Gradual reduction of hypoxic glow and pseudoflash intensity occurs in segment 7 until both are completely abolished after two hours, while the response in segment 6 can be maintained by mechanical irritation, eliciting

spontaneous flashes. In other cases, particularly in *Photinus,* the pseudoflash is immediately abolished in segment 7, except in one or two tiny spots which gradually lose the ability to generate pseudoflashes. Stimulation with electrodes placed dorsally in the seventh (denervated) segment partially restores the pseudoflash and hypoxic glow. The hypoxic glow can also be intensified by stimulation during hypoxia as in the normal light organ (Fig. 10).

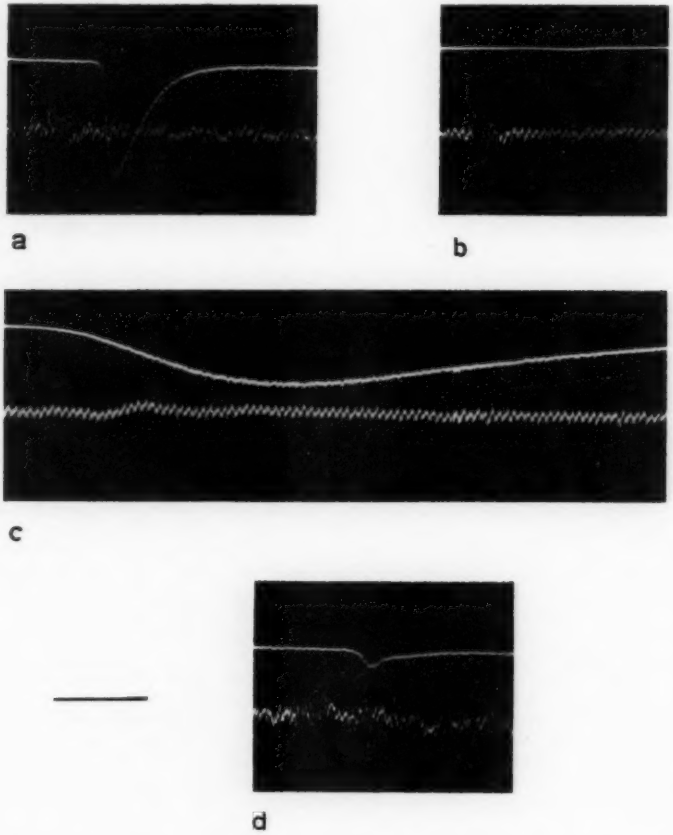

FIGURE 11. Recording of neural action potentials, spontaneous flashes, and pseudoflash in the lantern of an adult male, *Photuris pennsylvanica.* Photomultiplier output on upper trace (increasing intensity down). Action potentials on lower trace. Time base indicated by horizontal, dark line which represents 100 milliseconds. Time base reads from left to right. (a) Spontaneous flash and generating neural burst prior to hypoxia. (b) Extremely reduced spontaneous flash and preceding neural burst after hypoxia onset. (c) Pseudoflash induced with air. Neither neural nor muscle action potentials are seen. (d) Spontaneous flash and preceding neural burst following pseudoflash in air recovery period.

4. *Demonstration of lack of spontaneous neural activity during pseudoflash*

In order to further define the role of the nerves in pseudoflash generation, nerve action potentials were sought during the hypoxic period and pseudoflash by placing recording electrodes on the exposed ventral surface of the light organ. The results are illustrated in Figure 11. The animal was flashing spontaneously prior to the onset of hypoxia and before each flash a discrete burst of neural activity was noted (Fig. 11a). Immediately after onset of hypoxia two flashes of reduced magnitude were observed, each associated with an undiminished train of action potentials (Fig. 11b). Following these flashes no bursts or even isolated nerve impulses could be observed. Although no hypoxic glow was observed, a pseudoflash was produced without associated action potentials (Fig. 11c). Spontaneous flashes and associated neural bursts resumed immediately following the pseudoflash in air (Fig. 11d).

Discussion

These observations on active, quiescent, electrically stimulated and eserine-injected animals, and the results of nerve transection, strongly implicate the innervation in the pseudoflash. Our inability to detect nerve impulses during the hypoxic glow period, however, indicates that individual pseudoflashes in the intact animal can be induced independently of neural activity. One supposition seemingly warranted by the decline of successive pseudoflashes in quiescent animals is that previous neural activity serves as a priming mechanism. A priming activity with regard to spontaneous activity has been suggested by Case and Buck (1959).

The priming mechanism of neural activity on hypoxic glow and pseudoflash can be explained on the basis of the scheme of firefly luminescence developed by McElroy and his associates. Hypoxia may allow substrate accumulation from precursors in the intact animal just as it does *in vitro* as shown by Hastings, McElroy and Coulombre (1953), who were able repeatedly to induce flashes similar to pseudoflashes by alternating hypoxic and aerobic conditions until surface denaturation of the enzyme occurred. The reduction of intensity in successive pseudoflashes in our experiments would represent, according to this scheme, accumulation of an inhibitory complex. When pseudoflashes can no longer be induced, presumably all the enzyme is inhibited. As McElroy and Hastings (1955) speculated, one result of neural stimulation might be release of inorganic pyrophosphate which on the basis of *in vitro* observations would free the active enzyme and result in the ability to again induce high intensity pseudoflashes.

If nerves operate in fact as a priming mechanism by mediating destruction of an inhibitor, a number of observations of previous investigators could be explained. This action might explain why it is difficult or impossible to elicit pseudoflashes in quiescent animals since in them the active enzyme would be completely inhibited. This suggests that during the daily non-flashing periods the light organ would contain large amounts of this inhibitor, and neural activity would be low or absent. The great deal of areal variation observed in the pseudoflash response may simply be the result of spotty neural priming. This is supported by the observation that a total organ pseudoflash is usually obtained in actively flashing animals and by the observations that electrical stimulation during

hypoxia in quiescent animals commonly augments the hypoxic glow by recruiting new, discrete luminescing areas (Fig. 8).

Summary

1. Hypoxic glows and pseudoflashes were studied in adults of the lampyrid fireflies, *Photinus pyralis* Linn. and *Photuris* sp.

2. In spontaneously flashing *Photinus* adults a large number of successive pseudoflashes of high intensity can be induced. Induction of pseudoflashes in quiescent animals results in decline of successive pseudoflash and hypoxic glow intensities, and increase in the time required not only for hypoxic glow onset but also for development of maximal hypoxic glow intensity.

3. Electrical stimulation during hypoxia increases the rate of hypoxic glow rise, commonly by recruiting new luminescing areas within the lantern, and can maintain the hypoxic glow beyond the normal period. Electrical stimulation when continued during readmission of air superimposes flashes on the pseudoflash decay phase only.

4. Perfusion of the body cavity with 10^{-4} *M* eserine enhances the ability to induce high intensity pseudoflashes and hypoxic glows.

5. Transection of the nerves supplying the seventh abdominal segment reduces the ability of that segment to produce pseudoflashes, while pseudoflashes can be obtained from that portion of the lantern occupying the sixth segment. Electrical stimulation of the seventh segment after nerve transection can partially restore its pseudoflash capability.

6. No nerve impulses were observed during the hypoxic interval immediately prior to and during induction of the pseudoflash although neural bursts and their associated flashes were noted before and after hypoxia and during its early period.

7. The observations implicate the innervation in the pseudoflash. A possibility suggested is that previous neural activity serves as a priming mechanism by mediating the removal of a chemical inhibitor.

Literature Cited

Alexander, Robert S., 1943. Factors controlling firefly luminescence. *J. Cell. Comp. Physiol.*, **22**: 51–71.

Carlson, Albert D., 1959. Neural involvement in the firefly pseudoflash. *Biol. Bull.*, **117**: 407.

Case, James F., and John Buck, 1959. Central nervous aspects of firefly excitation. *Biol. Bull.*, **117**: 393.

Dahlgren, Ulric, 1917. The production of light by animals. *J. Franklin Inst.*, **183**: 323–348.

Hastings, J. Woodland, and John Buck, 1956. The firefly pseudoflash in relation to photogenic control. *Biol. Bull.*, **111**: 101–113.

Hastings, J. Woodland, William D. McElroy and Jane Coulombre, 1953. The effect of oxygen upon the immobilization reaction in firefly luminesence. *J. Cell. Comp. Physiol.*, **42**: 137–150.

McElroy, W. D., and J. W. Hastings, 1955. Biochemistry of firefly luminescence. Pp. 161–198 in The Luminescence of Biological Systems (Ed. F. H. Johnson), Amer. Assoc. Adv. Sci., Washington.

Roeder, K. D., 1953. Insect Physiology. John Wiley and Sons, Inc., New York.

Snell, Peter A., 1943. The control of luminescence in the male lampyrid firefly, *Photuris pennsylvanica*, with special reference to the effect of oxygen tension on flashing. *J. Cell. Comp. Physiol.*, **1**: 37–51.

EUGLENA GRACILIS IN SYNCHRONOUS DIVISION. II. BIOSYNTHETIC RATES OVER THE LIFE CYCLE

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Mapping of the major metabolic and synthetic events occurring over the course of the life cycle of single cells forms one of the principal endeavors of current physiological efforts, and very refined (albeit tedious) techniques have permitted notable successes in this area. However, the small size of growing cells imposes serious quantitative—and qualitative—limitations on the information to be gleaned from this approach, since many of the coarser techniques require masses several orders of magnitude above that provided by the single cell. Furthermore, natural variability of cells grown under identical conditions—best exemplified perhaps in the scatter of individual generation times (Powell, 1955; Prescott, 1959)—can often be extensive. The use of synchronously-dividing populations as a model of the single cell promises to surmount some of these difficulties, though new ones may well be introduced.

That populations of cells can be induced to divide rhythmically has been known for some time (von Denffer, 1949), but a clear appreciation of the possibilities offered by such systems appeared only within the last decade (Hotchkiss, 1954; James, 1954; Scherbaum and Zeuthen, 1954). It has already been made the subject of several review articles (Scherbaum, 1960; Zeuthen, 1958). Increased interest in this field has resulted in the elaboration of certain obvious restrictions which should be satisfied by the synchronously-dividing population before it may fairly be viewed as a working model of the single cell. For example, the time consumed in the burst of division activity should be relatively short compared to the over-all generation time; and the increase in population number occurring in each burst should be commensurate with the normal division behavior of member cells, if the division burst is to be equated to division of the single cell. These are but two of the more obvious parameters which characterize synchronous cell division. More subtle restrictions include continued and repetitive division bursts, *i.e.*, populations in which synchrony can be maintained indefinitely (as long as the environmental stimulus causing synchrony is continued) (Padilla and James, 1960). A first corollary of continued division synchrony is the "balanced growth" of all member cells, in the usage of Barner and Cohen (1956). A further restriction is always implied, namely that growth of member cells in the population be

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at least as synchronous as their division. Unfortunately, none of the cell types thus far synchronized can completely satisfy all these restrictions; as noted by Zeuthen (1958), however, even a modest degree of synchrony can be useful.

It is possible to synchronize division activity of free-living photosynthetic cells, at a constant temperature, merely by growing the cells autotrophically on light-dark cycles (Sweeney and Hastings, 1958: Tamiya *et al.*, 1953). If the light and dark periods are of appropriate lengths, the average cell grows uninterruptedly over one whole generation, and stores sufficient energy in the light period to satisfy demands for those processes culminating in cell division (Bernstein, 1960; Cook and James, 1960). In all photosynthetic systems so grown, the "burst" of division activity is confined for the most part to the dark period, so that growth and division are truly separated.

In populations of *Euglena gracilis,* the percentage of cells completing division in successive bursts gradually decreases with increased population density, presumably because mutual shading limits effective utilization of the photosynthetic apparatus. At population densities below about 5000 cells per ml., 90–95% of the cells complete division at each burst; if the culture is diluted once in each generation with fresh salt medium (at the temperature of the culture), so that the population density never approaches levels of the stationary phase, division synchrony can be maintained without abatement for long periods of time, perhaps indefinitely (see Figure 1). It can be seen from Figure 1 that divisions commence

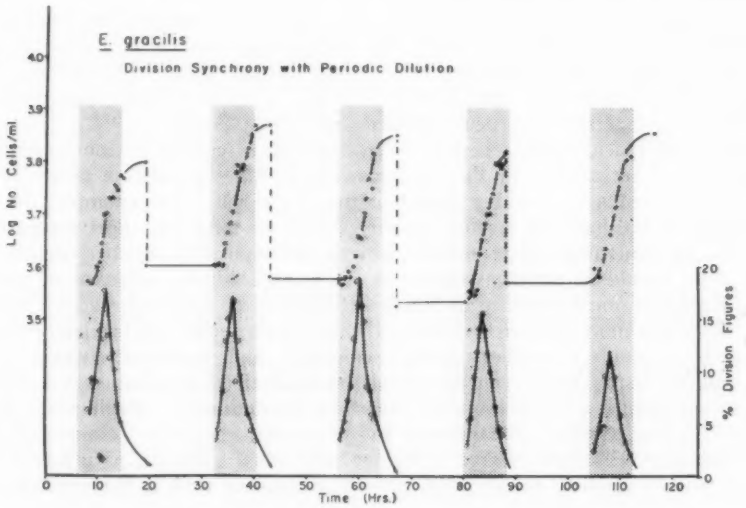

FIGURE 1. Light-induced division synchrony of *Euglena gracilis.* The upper curve is a plot of the logarithm (to the base 10) of the population density. The 8-hour dark periods, to which nearly all of the cell divisions are confined, are represented by the shaded areas. The light periods are of 16 hours duration. The broken lines indicate dilution with fresh salt medium at the temperature of the culture (20° C.). The lower curve shows the proportion of cells in recognizable fission.

at about the third hour of each dark period, and are terminated some two or three hours after the onset of the subsequent light period. Except for this very early portion, no cell divisions occur in the light period, which has a total duration of 16 hours. Of those cells completing division, 95% do so within a 6-hour span of time (James, 1960). Thus, about 75% of the total generation time is devoid of cell divisions, and most of this fraction is spent in the light, where major biosynthetic activities of *Euglena* should occur. Since the division synchrony of *Euglena gracilis* is well-defined and repetitive, the metabolic regulation of synthetic rates must be in fine adjustment at all ages, especially since the energy supplied (as light) is just that needed for growth and division of the average cell over one life cycle. Characterization of growth patterns in synchronized *Euglena* is therefore profitably made, and cultivation on a near-basal medium, where the photosynthetic apparatus itself must be duplicated, perhaps renders such a study all the more meaningful. Moreover, synchronous growth at a constant temperature obviates difficulties associated with the interpretation of temperature-sensitive rate processes, and thus circumvents some of the criticisms which have been leveled against other methods of synchronizing cell division (Abbo and Pardee, 1960; Mitchison and Walker, 1959; Prescott, 1960).

Mass increase and the distribution of volumes in member cells of synchronized *Euglena* populations have been reviewed in a previous paper, and the analogy of the population as a single cell discussed in some greater detail (Cook, 1961). While a wide range of cell volumes exists at any time, the (weighted mean) volume and dry mass exhibit a linear increase, to a doubling, over the whole of the 16-hour light period. The average *Euglena* in synchronous populations, then, is to a first approximation a successful working model of the single cell. The present report will describe growth patterns of other important cell parameters over the life cycle.

Methods and Materials

Culture methods have been described in detail previously (Cook and James, 1960). A constant-temperature water bath (American Instrument Co.) circulated water continuously through a thin transparent plastic jacket surrounding the cylindrical culture vessel, so that the culture was maintained at 20° C. An in-line reservoir of fresh medium, also kept at 20°, was used to dilute the culture once in each generation (see Figure 1), so that the population density at harvest always lay between 3000 and 5000 cells per ml., well below levels of the stationary phase. While this approach is only quasi-chemostatic, it is not likely that the medium ever became limiting for growth of *Euglena;* and in addition, changing characteristics of the average cell as a function of population density were avoided (Summers, 1960).

The medium was that of Cramer and Myers (1952), slightly modified (Padilla and James, 1960): $(NH_4)_2HPO_4$, 1.0 gm./L.; KH_2PO_4, 1.0 gm./L.; $MgSO_4 \cdot xH_2O$, 0.2 gm./L.; $CaCl_2$, 0.02 gm./L.; $Fe_2(SO_4)_3 \cdot xH_2O$, 3.0 mg./L.; $MnCl_2 \cdot 4H_2O$, 1.8 mg./L.; $CoSO_4 \cdot 7H_2O$, 1.5 mg./L.; $ZnSO_4$, 0.4 mg./L.; $Na_2MO_4 \cdot 2H_2O$, 0.2 mg./L.; $CuSO_4 \cdot 5H_2O$, 0.02 mg./L. These salts are chelated by sodium citrate (0.8 gm./L.), which is not used as a carbon source by *Euglena.* The vitamins B_1 (0.1 mg./L.) and B_{12} (0.0005 mg./L.) are required by *Euglena,* and were added aseptically to the autoclaved salt-citrate solution after sterilization by filtration. The phosphates buffer the medium to a pH of 6.8. In this medium, $(NH_4)_2HPO_4$

is the sole nitrogen source. However, small amounts of the sulfhydryl-bearing amino acids methionine (10^{-5} *M*) and cysteine hydrochloride (6.4×10^{-4} *M*) were routinely added to the medium (after sterilization by filteration). While these concentrations do not support growth, they can effectively improve division characteristics of *Euglena* in synchronous culture (Cook and James, 1960; James, 1960). Incandescent lamps (General Electric Co.), with an incident intensity of 130 foot-candles, were the sole energy source, and nutrition, save for catalytic amounts of the vitamins and two amino acids, was strictly autotrophic. In continuous light at 20° C., this intensity is saturating for growth of *Euglena*.

Cultures were continuously stirred, by means of a teflon-covered magnetic impeller bar, to ensure even illumination and rapid exchange of gases. Harvests were made by means of a gravity-flow siphon, and stirring further served to randomize the distribution of cells within the culture. After the syphon was flushed, all aliquots were representative of the population as a whole.

Cultures were axenic, and were kept on the light-dark cycle from inoculation. Dilution of the medium was initiated when the appropriate population density was reached, and repeated once in each generation; harvests of any given culture were deferred until at least five light-dark cycles had elapsed after the first dilution. Generally, two light-dark cycles suffice for the induction of division synchrony, and initial harvests were delayed only as a precaution against the possible tardy stabilization of other cellular parameters.

All cell counts were made with the Coulter cell counter (Coulter Co.). Details of this procedure can be found elsewhere (Mattern *et al.*, 1957); the mean values of replicate counts made with this instrument are within 2% of the true value when no correction for coincidence is necessary. For the present case, aliquots were always diluted to cell densities yielding a linear counting rate, so that coincidence was negligible.

At harvest, cells were first packed by gentle centrifugation, and then resuspended to densities appropriate to the analytical procedure being followed. Representative aliquots of this concentrated suspension were then diluted with the salt medium for cell counts.

Total protein content of the average cell was estimated by the Folin-Ciocalteau phenol test as modified by Lowry *et al.* (1951), after prior extraction of pigments and other soluble fractions with 90% aqueous acetone. Bovine serum albumin in known concentrations was simultaneously run as a standard, and protein levels in *Euglena* are expressed in these equivalents.

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted according to the method of Schmidt and Thannhauser (1945). Optical densities of the hydrolyzed DNA and RNA (in 5% perchloric acid) were measured at 260 and 315 mμ with the Beckman DU spectrophotometer. Regression curves obtained with serial concentrations of commercial DNA and RNA permitted conversion of the observed difference reading ($O.D._{260} - O.D._{315}$) into absolute amounts per cell.

Pigments of the photosynthetic apparatus were estimated according to the method of Richards with Thompson (1952). After overnight extraction of a known number of cells in 90% aqueous acetone at 15° C., the initial extract was made up to volume (10 ml.) with two washes. Optical densities were determined in the Beckman DU spectrophotometer at wave-lengths of 750, 665, 645, 630, 510,

and 480 mμ, with 90% acetone as a blank. Pigments in *Euglena* include the chlorophylls *a* and *b*, and several carotenoids, of which beta-carotene is the most important in terms of absolute amounts (Fogg, 1953). None of these absorb appreciably at 750 mμ, and readings at this wave-length (usually negligible) were routinely subtracted from those at the other wave-lengths as a correction for turbidity. Simultaneous equations developed by Richards with Thompson (1952) permit calculation of pigment concentrations from the observed optical densities. For the chlorophylls, concentrations in absolute amounts are obtained. The method serves as a satisfactory measure of chlorophyll *a* in *Euglena*. Chlorophyll *b* is present in much smaller amounts, however (about 10% that of chlorophyll *a*). This pigment is therefore largely masked by the other pigments, and accurate measurements are not feasible by this method. Since the several carotenoids are estimated as a mixture, concentrations must be expressed in arbitrary units. Richards with Thompson have proposed the Standard Pigment Unit (SPU) for this measure, and this unit is retained here. Their equations are satisfactory for estimates of carotenoid content in *Euglena* (Cook, 1960).

The method of cultivating cells which was adopted for these studies limited the working volume of the culture to about one liter per day, *i.e.*, a yield of no more than 5×10^6 cells in each generation. Since some of the procedures required almost this many cells for a single determination, a complete picture of synthetic rates could

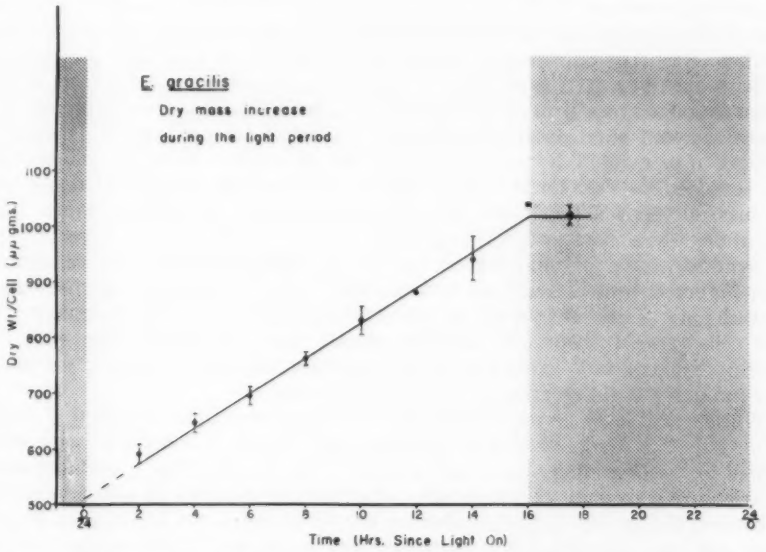

FIGURE 2. Dry mass of the average cell at different times during any given light-dark cycle. The abscissa is the time scale for one complete cycle, beginning arbitrarily with the onset of the 16-hour light period. The shaded area at the left represents the end of the preceding dark period, and that at the right the whole 8-hour dark period for the cycle shown. Limits of one standard deviation are indicated.

be compiled only by pooling data from many different light-dark cycles. Continued synchronous culture by periodic dilution, as illustrated in Figure 1, thus served practical as well as theoretical reasons. In the following, statistical estimates of experimental error are shown as the standard deviation about the mean, and represent both errors of analysis and of cell counts.

Unfortunately, the cell used in these studies was previously referred to as *Euglena gracilis* var. *bacillaris* (Cook and James, 1960). Certain characteristics, not discovered until after that paper had been accepted for publication, made it clear that the cell was not in fact *E. gracilis* var. *bacillaris,* but another strain of the species. Pending positive identification, the cell is called merely *Euglena gracilis.*

Results

Figure 2 shows the dry weight of the average *Euglena,* measured at different times during the light-dark cycle. These data have been reviewed before (Cook, 1961), and are shown here for reference. The dark period is represented by the shaded portion, and the abscissa is the time-scale for one complete cycle—24 hours—beginning arbitrarily with the onset of the light period. The rate of dry mass increase is clearly linear over the whole of the light period, and is terminated when the cells enter the dark period. Since cell divisions are still occurring in the first two hours of the light period, meaningful mass measurements on a per cell basis are not easily made at this time. It seems fair to estimate mass changes in the early hours of the light period by extrapolation from the rate observed after cell divisions are complete. The extrapolated portion of the rate curve in Figure 2 is indicated by the broken line. (This treatment ignores the possibility of a brief lag in mass accumulation very early in the light period.) Since *Euglena* essentially doubles in number at each burst of division activity, continued balanced growth demands a duplication of major cell constituents over the life cycle. Figure 2 indicates that this is indeed the case for the dry weight of *Euglena;* the average filial cell has a dry mass of about 500 $\mu\mu$grams, and the average parent cell 1000 $\mu\mu$grams. Mass loss in the 8-hour dark period is negligible.

Synthetic rates for total protein are not so straightforward. As can be seen from Figure 3, protein synthesis is terminated (after a doubling) at the tenth or eleventh hour of the light period, at a level which remains constant thereafter. The filial cells contain about 200 $\mu\mu$grams protein, and the parent cells 400. The protein : dry mass ratio is 0.4 both at the beginning and end of the light period, but goes through a slight maximum (0.5) in the interim.

Nucleic acid levels in the average cell were determined less frequently. Figure 4 shows the RNA content at four different ages. If the rate curve is again extrapolated to the beginning of the light period, it can be seen that a doubling occurs linearly during the entire light period. The filial cell contains 23 $\mu\mu$grams, and the parent cell 46 $\mu\mu$grams. Less confidence can be placed in the DNA data, and this study will bear repetition with culture conditions yielding larger numbers of cells for this analysis. However, Figure 5 indicates that DNA is probably doubled in a linear fashion throughout the light period.

Duplication of chlorophyll *a* is completed by the fourteenth hour of the light period, after which no further increase occurs (Fig. 6). Similarly, carotenoid synthesis (in *Euglena,* mostly beta-carotene) is terminated about two hours before

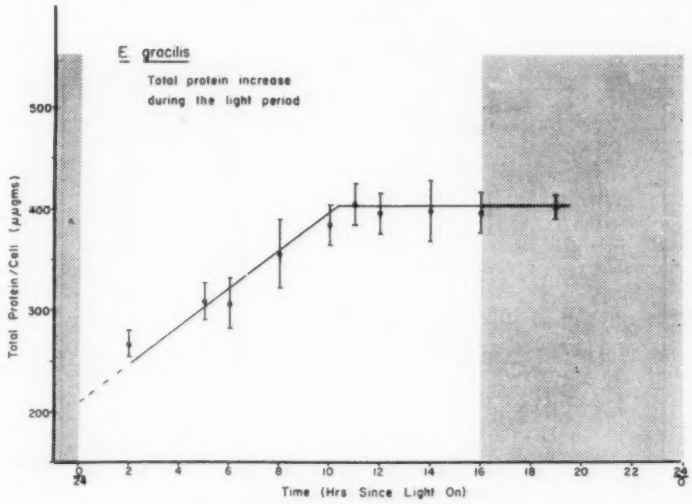

FIGURE 3. Total protein of the average *Euglena* at different times during any given light-dark cycle.

the onset of the dark period (Fig. 7). The ratio carotenoids : chlorophyll *a* is thus constant throughout the life-cycle of the average cell. Chlorophyll *a* contributes about 3% to the total dry weight of *Euglena*, although this value goes through a slight maximum at the fourteenth hour of the light period. From data of Goodwin and Jamikorn (1954), it can be calculated that the carotenoids in synchronized

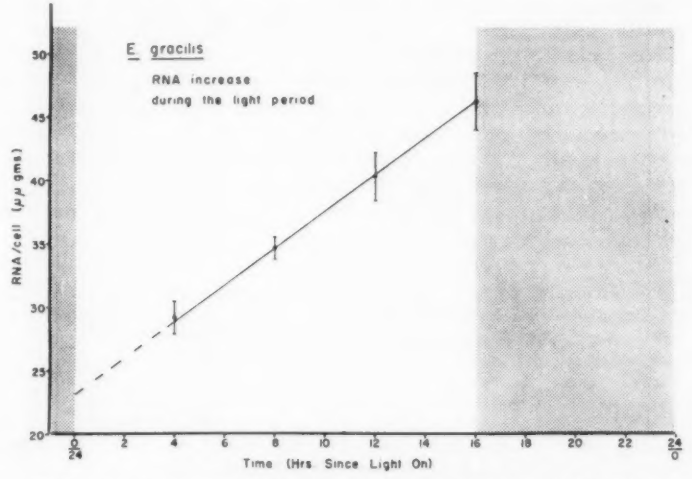

FIGURE 4. RNA synthesis by the average cell during the light period.

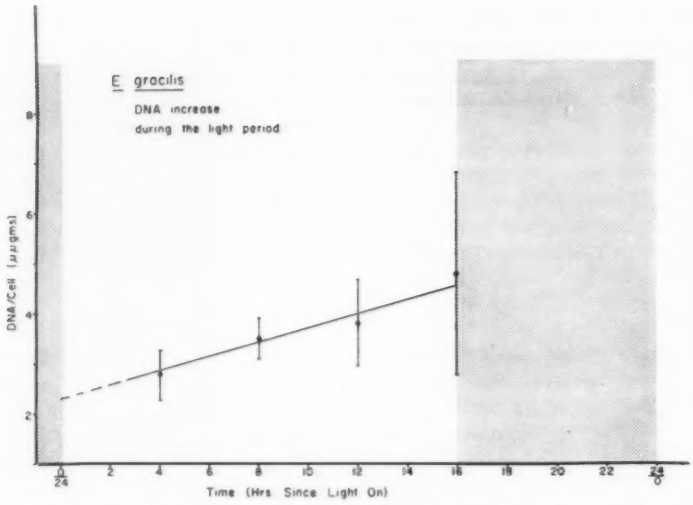

FIGURE 5. DNA content of the average *Euglena* during the light period.

Euglena constitute one to two per cent of the total dry weight (Cook, 1960), and this, too, goes through a slight maximum in the light period.

Table I lists the concentrations of chlorophyll *b* calculated for different ages. While there is considerable error involved in estimates of this pigment by the method used here, the expected two-fold difference in chlorophyll *b* levels should at least

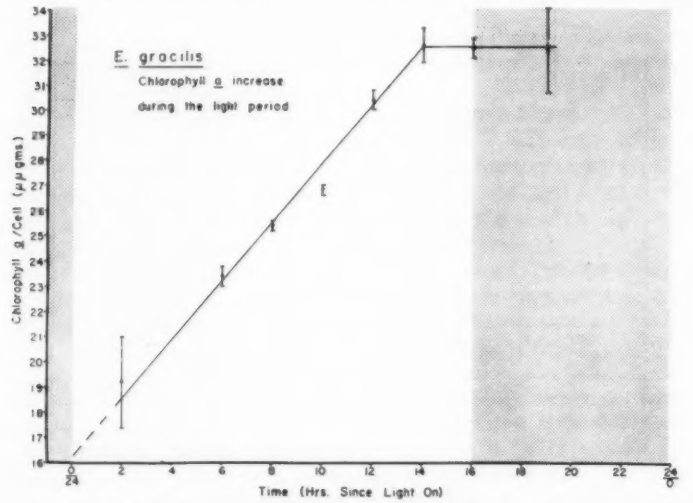

FIGURE 6. Chlorophyll *a* content of the average cell during any given light-dark cycle.

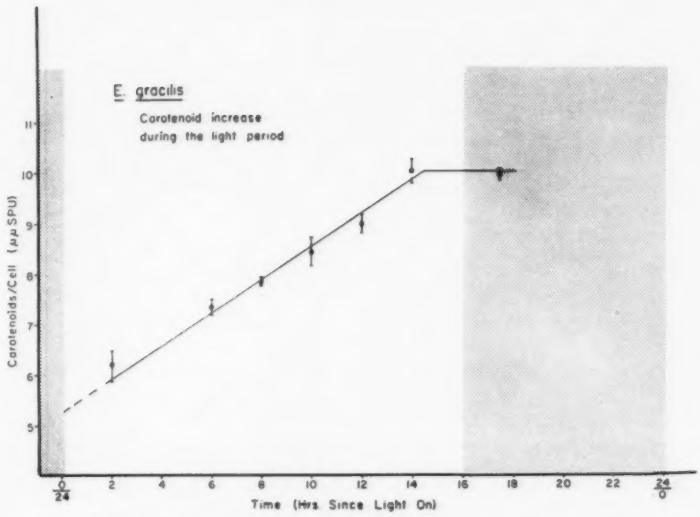

FIGURE 7. Carotenoid synthesis by the average *Euglena*.

be indicated; that is to say, even rough approximations should reveal some tendency toward increasing amounts if chlorophyll *b* is indeed duplicated in the light period. No such differences are seen over the major portion of the light period, however; at the sixth hour, the average cell has 2.60 ± 0.38 μμgrams chlorophyll *b*, and at the onset of the dark period, 2.67 ± 0.24 μμgrams. It is not at all unlikely that chlorophyll *b* is duplicated in the very early portion of the light period. The uncertain role of chlorophyll *b* in the family of photosynthetic pigments need not after all demand a constant ratio of chlorophyll *a*:chlorophyll *b*. Thus, Smith (1949) and Koski (1950) have shown that the biosynthesis of chlorophylls *a* and *b* in etiolated seedlings proceeds at different and apparently unrelated rates. For *Euglena*, a more sensitive analysis than that used here must be adopted before accurate rate measurements of chlorophyll *b* synthesis can be known. The possibility that a continuously changing ratio of photosynthetic pigments occurs over the life cycle of *Euglena* implies a regulation of metabolism which could be of first importance to the over-all balanced growth of the cell.

TABLE I

Chlorophyll b *content of* Euglena gracilis *in synchronous culture, calculated according to the equations of Richards with Thompson (1952). The numbers following "Age" represent the time in hours following onset of the 16-hour light period.*

Age	2	6	8	10	12	14	16	17.5	19
Chlorophyll *b* (μμgm./cell)	1.87 ±.42	2.60 ±.38	2.53 ±.08	2.44 ±.06	2.79 ±.22	3.21 ±.21	2.67 ±.24	2.86 ±.14	3.11 ±.45

Discussion

The energy supply supporting cell division, an event which comes in the dark period in synchronous populations of *Euglena gracilis,* as well as maintenance of the cell in this period, must ultimately be derived from photosynthetic products elaborated in excess of immediate needs for incremental growth, and stored up during the light period against future requirements of the dark period. In point of fact, an estimate of the quantity of this stored energy was the basis leading to the particular light-dark cycle on which *Euglena* is grown (Cook and James, 1960). One might therefore have expected the total dry mass of the average cell to somewhat more than double during active synthesis in the light period. Figure 2 indicates that this is not the case, however. Each parent cell of 1000 $\mu\mu$grams dry weight yields two filial cells of 500 $\mu\mu$grams (assuming equal division), and each of these enters the subsequent light period with just this weight. Thus, parent and filial cells traverse the entire 8-hour dark period with no significant reduction of mass.

Cramer and Myers (1952) have estimated that *Euglena gracilis* consumes twice its own volume of oxygen every hour. While respiratory rates of *Euglena* in synchronous culture have not yet been determined, it is probable that similar rates will be found. With this assumption, and with the further assumption that an RQ of 1 obtains, it can be calculated that the total carbon lost (as CO_2) in the dark period is only 35 $\mu\mu$grams for the average parent and generated filial cells. This is less than 4% of the total dry weight, not likely to be detected by a technique which has a probable error of about 5%.

In most euglenoid flagellates, protein constitutes about 30–40% of the total dry weight (Cook, 1960). In autotrophic strains, photosynthetic pigments contribute about 5%, and the nucleic acids 4 or 5%. Most of the remaining dry mass, some 50%, will be made up of storage products—lipids and paramylum, the characteristic polysaccharide of these cells (Kudo, 1954). Although these storage products were not measured over the life cycle of *Euglena,* it is inferred that their accumulation must complement protein synthesis in such a way that the over-all increase in dry mass, of which these fractions make up the bulk, follows the linear rate shown in Figure 2. The ratio of storage products to dry mass therefore probably passes through a minimum at the same time of the light period as the protein : dry weight ratio exhibits a maximum.

Major synthetic patterns of the average cell in synchronized populations of *Euglena gracilis,* growing autotrophically on a simple, near-basal salt medium at 20° C., are summarized in Figure 8. The ordinate is in relative units, from 1 to 2, the range of increase in these parameters expected for the single cell over its life cycle. The time scale on the abscissa is shifted somewhat from that seen in the earlier graphs, with respect to the light-dark cycle, to more nearly accommodate the actual age of the average cell. This cell has its inception, by division of the parent, some two hours before the onset of the light period, and 24 hours later is itself the parent of two filial cells, an event which occurs at the sixth hour of the subsequent dark period, being preceded by a mitosis which is probably of three or four hours duration (Leedale, 1958). Most of the interphase is spent in the light, where the cell completes its physical growth and duplicates most (if not all) of its principal biochemical constituents. Increase in dry mass is limited to the light period, and follows a linear rate over the entire 16 hours, as does the cell volume (Cook, 1961).

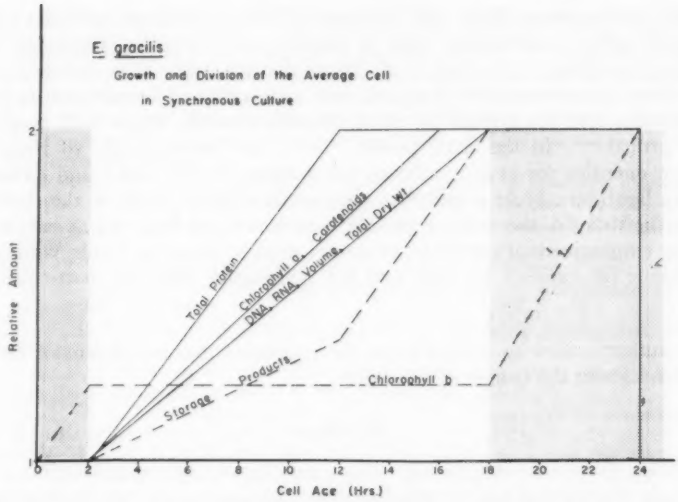

FIGURE 8. Summary of biosynthetic rates of major constituents in the average *Euglena* during any given light-dark cycle. The ordinate shows the relative content, the range of values observed in duplication of the various quantities listed (Figs. 2–7). The broken lines are inferred rates (see text). The average cell is formed by division of a parent about two hours before the onset of the light period (age 0) and divides at about the sixth hour of the subsequent dark period (age 24) to yield two filial cells.

RNA is similarly doubled, and probably also DNA. Protein synthesis, though limited to the light period, is complete by the twelfth or thirteenth hour of the cell's life; storage products, which make up most of the remaining dry mass, are most likely accumulated so as to complement protein synthesis (Fig. 8, broken line). Chlorophyll *a* and the several carotenoids are synthesized only in the light, and are duplicated by the fourteenth hour of this period, when the cell is 16 hours old. Chlorophyll *b* synthesis (Fig. 8, broken line) may occur in the dark period, but the possibility of duplication very early in the light period is not to be excluded. Certainly, most of the cell's growth is confined to the light period.

Division of the average cell, on the other hand, comes in the dark. With well-defined synchronous systems, the average cell is not likely to divide before growth has been completed, and for the present case this must of necessity follow the light period. Moreover, it has been shown that visible light can inhibit cell division in the euglenoid flagellates (Cook, 1960; Leedale, 1959), and division of even mature euglenoid cells can be delayed by light for as much as 20% of their normal generation time. A repetitive cycling of metabolic events, such as the growth patterns described in this paper, coupled with a periodic inhibition of cell division, are doubtless additive in their effect on the continued synchronous divison of *Euglena gracilis*. While photosynthetic cells can of course grow and multiply in continuous light, it is likely that light can be used more effectively for growth (and division) when punctuated periodically by dark periods. Sorokin and Krauss (1959), for example, have reported that *Chlorella* has a higher growth rate with intermittent periods of

dark than in continuous light, and Warburg (1957) has commented that culture of autotrophic cells in continuous light is contrary to conditions demanded by their biochemical evolution. Implicit in all this is the possibility that synthesis of minor cell fractions in synchronized *Euglena*, not necessarily of importance in terms of absolute mass, but yet critical for most effective growth, might well occur with a greater probability in the dark period. It is not unreasonable to imagine that activation energies for such syntheses are reduced in the dark; and culture on a repetitive light-dark cycle would then soon confine these events to the dark period. The varying ratios of the major metabolic constituents of *Euglena*, as seen in Figure 8, further emphasize that metabolic processes need be integrated only with reference to the whole life-cycle of the cell, and not necessarily with any particular portion thereof.

The author wishes to acknowledge the continued encouragement of Dr. T. W. James throughout the course of this work.

Summary

1. When grown autotrophically on an appropriate light-dark regimen, populations of the flagellate *Euglena gracilis* divide synchronously, an approximate doubling of cell number occurring in each dark period.

2. Growth of the average cell during the light period, which is essentially devoid of cell divisions, is described in terms of dry mass, total protein, DNA, RNA, chlorophyll *a*, and the carotenoids. The average *Euglena* duplicates all of these constituents in each light period.

3. Dry mass, volume, RNA, and probably DNA of the average cell increase to a doubling in a linear fashion over the whole of the 16-hour light period.

4. The photosynthetic pigments chlorophyll *a* and the carotenoids (beta-carotene) increase at rates which do not deviate greatly from linearity.

5. Synthesis of total protein is completed 5 or 6 hours before termination of the 16-hour light period. It is inferred that storage products are synthesized at rates complementing protein synthesis in such a way that dry mass increases linearly.

LITERATURE CITED

Abbo, F. E., and A. B. Pardee, 1960. Synthesis of macromolecules in synchronously dividing bacteria. *Biochim. et Biophys. Acta*, **39**: 478–485.

Barner, H. D., and S. S. Cohen, 1956. Synchronization of division of a thymineless mutant of *Escherichia coli*. *J. Bacteriol.*, **72**: 115–123.

Bernstein, E., 1960. Synchronous division in *Chlamydomonas moewusii*. *Science*, **131**: 1528–1529.

Cook, J. R., 1960. Light-induced division synchrony of *Euglena gracilis*, and the effects of visible light on related cells. Ph.D. Dissertation. University of California, Los Angeles.

Cook, J. R., 1961. *Euglena gracilis* in synchronous division. I. Dry mass and volume characteristics. *Plant and Cell Physiol.*, **2**: 199–202.

Cook, J. R., and T. W. James, 1960. Light-induced division synchrony in *Euglena gracilis* var. *bacillaris*. *Exper. Cell Res.*, **21**: 583–589.

Cramer, M., and J. Myers, 1952. Growth and photosynthetic characteristics of *Euglena gracilis*. *Archiv. f. Mikrobiol.*, **17**: 384–402.

von Denffer, D., 1949. Die planktische Massenkultur pennater Grunddiatomeen. *Archiv. f. Mikrobiol.*, **14**: 159–202.

FOGG, G. E., 1953. The Metabolism of Algae. John Wiley and Sons, Inc., New York.

GOODWIN, T. W., AND M. JAMIKORN, 1954. Studies in carotenogenesis. Some observations on carotenoid synthesis in two varieties of *Euglena gracilis*. *J. Protozool.*, **1**: 216–219.

HOTCHKISS, R. D., 1954. Cyclical behavior in pneumococcal growth and transformability occasioned by environmental changes. *Proc. Nat. Acad. Sci.*, **40**: 49–55.

JAMES, T. W., 1954. Quantitative studies on the ribonucleic acid content in *Amoeba proteus* in relation to the nucleus and the division cycle. Ph.D. Dissertation. University of California, Berkeley.

JAMES, T. W., 1960. Controlled division synchrony and growth in protozoan micro-organisms. *In:* Second Conference on Cell Division, New York Acad. Sci., **90**: 550–564.

KOSKI, V. M., 1950. Chlorophyll formation in seedlings of *Zea mays* L. *Arch. Biochem.*, **29**: 339–343.

KUDO, R. R., 1954. Protozoology. Fourth edition, Charles C Thomas, Publisher, Springfield.

LEEDALE, G. F., 1958. Mitosis and chromosome numbers in the Euglenineae (Flagellata). *Nature*, **181**: 502–504.

LEEDALE, G. F., 1959. Periodicity of mitosis and cell division in the Euglenineae. *Biol. Bull.*, **116**: 162–174.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

MATTERN, C. F. T., F. S. BRACKETT AND B. J. OLSON, 1957. Determination of number and size of particles by electrical gating: blood cells. *J. Applied Physiol.*, **10**: 56–70.

MITCHISON, J. M., AND P. M. B. WALKER, 1959. RNA synthesis during the cell life cycle of a fission yeast *Schizosaccharomyces pombe*. *Exper. Cell Res.*, **16**: 49–58.

PADILLA, G. M., AND T. W. JAMES, 1960. Synchronization of cell division in *Astasia longa* on a chemically defined medium. *Exper. Cell Res.*, **20**: 401–415.

POWELL, E. D., 1955. Some features of the generation times of individual bacteria. *Biometrika*, **42**: 16–44.

PRESCOTT, D. M., 1959. Variations in the individual generation times of *Tetrahymena geleii* HS. *Exper. Cell Res.*, **16**: 279–284.

PRESCOTT, D. M., 1960. Relation between cell growth and cell division. IV. The synthesis of DNA, RNA, and protein from division to division in *Tetrahymena*. *Exper. Cell Res.*, **19**: 228–238.

RICHARDS, F. A., WITH T. G. THOMPSON, 1952. The estimation and characterization of plankton pigments by pigment analysis. II. A spectrophotometric method for the estimation of plankton pigments. *J. Marine Res.*, **11**: 156–172.

SCHERBAUM, O. H., 1960. Synchronous division of microorganisms. *Ann. Rev. Microbiol.*, **14**: 283–310.

SCHERBAUM, O., AND E. ZEUTHEN, 1954. Induction of synchronous cell division in mass culture of *Tetrahymena pyriformis*. *Exper. Cell Res.*, **6**: 221–227.

SCHMIDT, G., AND S. J. THANNHAUSER, 1945. A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.*, **161**: 83–89.

SMITH, J. H. C., 1949. Processes accompanying chlorophyll formation. *In:* Photosynthesis in Plants, Iowa State College Press, Ames, Iowa.

SOROKIN, C., AND R. W. KRAUSS, 1959. Maximum growth rates in synchronized cultures of green algae. *Science*, **129**: 1289.

SUMMERS, L., 1960. The physiological variation of cell and nuclear volumes. Ph.D. Dissertation. University of California, Los Angeles.

SWEENEY, B. M., AND J. W. HASTINGS, 1958. Rhythmic cell division in populations of *Gonyaulax polyedra*. *J. Protozool.*, **5**: 217–224.

TAMIYA, H., K. SHIBATA, T. SASA, T. IWAMURA AND Y. MORIMURA, 1953. Effect of diurnally intermittent illumination on the growth and some cellular characteristics of *Chlorella*. *In:* Algal Culture from Laboratory to Pilot Plant (ed. J. S. Burlew), Carnegie Institution of Washington Publication 600, Washington, D. C.

WARBURG, O., 1957. Photosynthesis. *Angewandte Chemie*, **69**: 627–634.

ZEUTHEN, E., 1958. Artificial and induced periodicity in living cells. *In:* Advances in Biological and Medical Physics, vol. VI, Academic Press Inc., Publishers, New York.

OSMOTIC TOLERANCE AND REGULATION IN CRABS FROM A HYPERSALINE LAGOON

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It has been well established that the semi-terrestrial crab *Pachygrapsus crassipes* can osmo-regulate strongly in both dilute and concentrated sea water (Jones, 1941; Prosser *et al.*, 1955; Gross, 1955). On the other hand *Hemigrapsus oregonensis,* which is a less terrestrial crab than *Pachygrapsus,* was shown by Jones (1941) to be a strong hyper-osmotic regulator (maintenance of blood concentration above the concentration of the external medium), but was incapable of hypo-osmotic regulation in concentrated sea water after an immersion period of 72 hours. Gross (1957a), however, demonstrated hypo-osmotic regulation for *Hemigrapsus* in 150% sea water for a period of about 20 hours. The osmotic behavior of these two crabs fits well into the correlation between terrestrial behavior and hypo-osmotic regulation first indicated by Jones (1941) and expanded by Gross (1955). That is, crabs tending toward life on land are able to regulate in concentrated sea water and this in turn is accompanied by the additional ability to regulate in dilute sea water.

The adaptive significance of osmotic regulation in terrestrial and semi-terrestrial crabs was questioned by Gross (1955), inasmuch as adult land crabs will drown when completely immersed. Also, the semi-terrestrial *Pachygrapsus* is but rarely found in salinities deviating much from normal and, in fact, shows a precise preference for normal sea water. Thus, this highly active crab will avoid osmotic stress and is capable of seeking the normal salinities of the open sea (Gross, 1957b).

In January of 1959, Los Penasquitos lagoon, near San Diego, California, became isolated from the open sea by a sand bar. In early summer it was observed that large populations of *Pachygrapsus crassipes* and *Hemigrapsus oregonensis* and a relatively small population of the fiddler crab, *Uca crenulata,* were trapped but thriving in the lagoon where salinities were in excess of 150% sea water (5.3% salt). This was the first situation in which the author had found *Pachygrapsus* in the field where it was exposed to osmotic stress yet could not reach normal sea water. Although a complete field study of the environmental salinities of *Uca crenulata* has not been made, it is known to be a strong hypo-regulator (Jones, 1941) and is commonly known to live in back-bay areas where high salinities would be expected. On the other hand, the presence of *Hemigrapsus* in a hyper-saline lagoon was indeed surprising and suggested that contrary to laboratory findings was probably demonstrating hypo-regulation of significant magnitude and duration.

This investigation is concerned with ionic and osmotic regulation in *Hemigrapsus* and *Pachygrapsus* collected from Los Penasquitos lagoon during the period from July 1, 1959, to December 2, 1960, when the lagoon remained isolated from the open

sea. Physical and other biological aspects of the lagoon during parts of this period are presented by Carpelan (1961).

Materials and Methods

Blood and urine were sampled from the crabs as previously described (Gross, 1959). In the field blood and urine were taken from the animals immediately after capture. The samples were then placed into cold vials and quick-frozen on dry-ice. Also, sea water samples were taken at the site of capture and from different depths as a check for stratification. All samples were then returned to the laboratory for analysis. Salinity was determined by conductivity, using a 1000-cycle bridge. Na and K were determined by flame photometry; Ca and Mg by titration with ethylene diamine tetra acetic acid (EDTA) as previously described by Gross (1959). Melting points of blood and urine were made by the method of Gross (1954). Salinity preference was determined by means of a selectivity chamber (Gross, 1957b).

Results

Figure 1 presents the blood concentrations of both *Pachygrapsus* and *Hemigrapsus* sampled in the field at various dates and salinities. Blood concentrations (% sea water) are represented by: (a) total cations (mM/l.) (Na, K, Ca and Mg) relative to those found in normal sea water, and (b) by melting points. For seventeen crabs on which both blood melting point and total cations were determined, the ratio, $\frac{\text{melting point (\% sea water)}}{\text{total cations (\% sea water)}} = 0.98$, S.D. = 0.006. Thus, total cations (mM/l.) can be converted with sufficient precision to melting point and either type of determination will show the degree of osmotic regulation demonstrated by the crabs. Although the salinities of the lagoon water differed from site to site, those indicated in Figure 1 represent the media from which the crabs were actually collected.

It thus can be seen that *Hemigrapsus* (indicated by circles) was indeed regulating osmotically in the hypersaline water. Regulation was particularly strong in 160% sea water, but some *Hemigrapsus* were maintaining the blood concentration considerably below the ambient medium of 175% sea water (August 25, 1959). When the lagoon water reached approximately 180% sea water in mid-September, 1959, hypo-osmotic regulation in *Hemigrapsus* had essentially broken down; only one crab could be considered to be regulating with any strength. It also should be noted that the blood of several crabs was above the concentration of the indicated medium. This perhaps can be explained by the fact that most crabs observed (both species) at this time were piled up at the interphase between air and water, having migrated out of the depths of the lagoon. It is, therefore, likely that crabs captured when immersed had been out of the water and exposed to evaporation which had in turn elevated the blood osmotic pressure. While there were many dead and moribund *Hemigrapsus* at the edges of the lagoon at this time, only active specimens were sampled.

When the lagoon water reached a concentration of about 190% sea water no living *Hemigrapsus* could be found, although *Pachygrapsus* (indicated by triangles) were still surviving and active in these salinities. It cannot be concluded from these

data, however, that the upper limits of salinity in which *Hemigrapsus* can survive in nature lie somewhere between 180% and 190% sea water because there are many other physical and biological factors involved.

As was expected some *Pachygrapsus* were demonstrating strong hypo-regulation in all the salinities indicated in Figure 1.

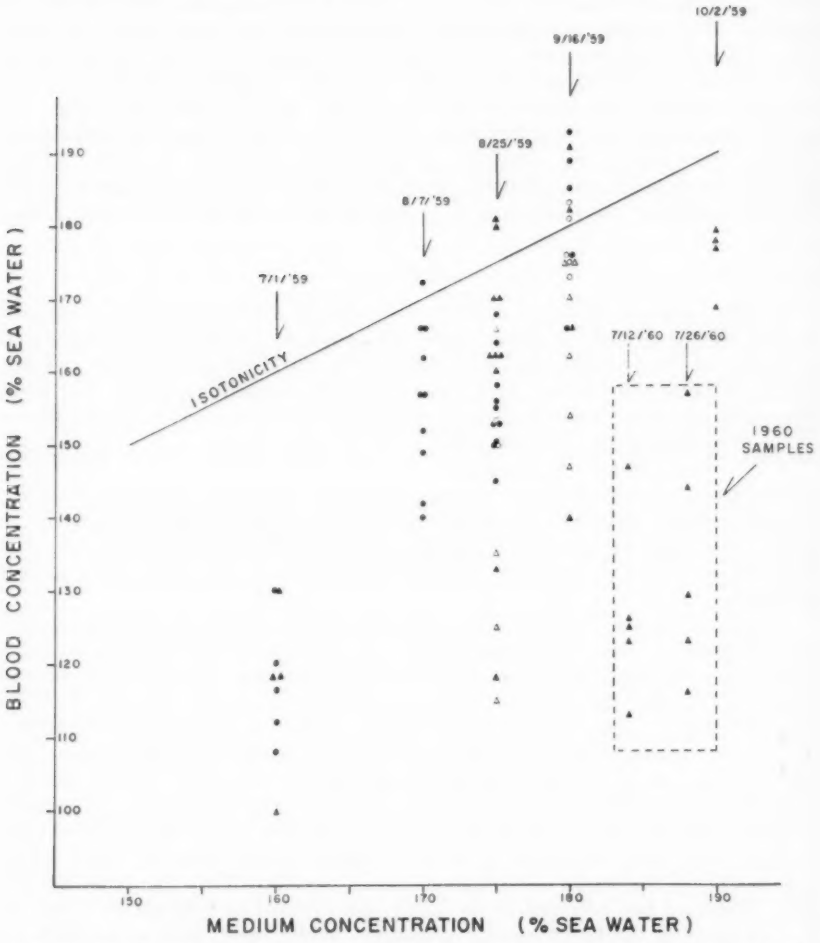

FIGURE 1. Osmotic regulation and tolerance by *Pachygrapsus* and *Hemigrapsus* in Los Penasquitos lagoon. Blood concentrations for individual crabs are represented by circles for *Hemigrapsus;* by triangles for *Pachygrapsus.* Each point represents a different individual. Blood concentrations by melting point determination are indicated by open points; blood concentrations by total cations (mM/l.) are indicated by solid points. Dates of sampling are indicated by arrows. The 1960 samples are enclosed by broken line.

An interesting phenomenon demonstrated by both species in Figure 1 is their ability to tolerate high blood concentrations. Thus, active *Hemigrapsus* and *Pachygrapsus* had blood concentrations which were close to isotonic or hypertonic to 180% sea water.

In December, 1959, the lagoon was filled by the high tides and the salinity dropped to 109% sea water although the isolating bar remained intact. However, no living *Pachygrapsus* or *Hemigrapsus* could be found. Nevertheless, in the summer of 1960, both *Pachygrapsus* and *Hemigrapsus* were found in relatively small numbers in the isolated lagoon which again had become hypersaline. The specimens of *Hemigrapsus* were sufficiently small to have washed over the bar during the winter high tides in the larval stage and developed in the lagoon. Most *Pachygrapsus* were mature and therefore either entered the lagoon across the bar in the adult stage from a sandy beach not likely to be inhabited by this species or were remnants from the 1959 population. Blood concentrations of the 1960 *Pachygrapsus* samples (enclosed by broken line in Figure 1) show that hypo-osmotic regulation was stronger in the 1960 crabs than in the 1959 crabs. The combined 1960 blood samples taken from animals immersed in salinities greater than 180% sea water concentrations were lower in concentration on the average than the 1959 samples taken from crabs immersed in 175% sea water, $P < 0.01$. This suggests that rigorous selection had taken place which favored hypo-regulation in *Pachygrapsus,* and that the 1960 specimens were survivors of this selection from the 1959 population. As indicated above, the salinity of the lagoon water in early 1960 was close to normal. Thus, the 1960 crabs were not regulating more strongly than those collected in 1959 because of long-term acclimatization, although two successive seasons of exposure to increasing salinities might have had the same effect.

While immature *Hemigrapsus* were captured from the lagoon in 1960 when the maximum salinity was 188% sea water, samples of sea water from the sites of capture were not taken. Thus, the degree of regulation for this species cannot be told for 1960. It is interesting, however, that two pooled samples of blood from these small specimens were approximately isotonic to the maximum salinity found in the lagoon at that time (188% sea water).

It is apparent from the data in Figure 1 that contrary to laboratory finding (Jones, 1941; Gross, 1957a) *Hemigrapsus* in nature is quite capable of strong hypo-osmotic regulation. The contradiction between laboratory studies and the field studies of the present investigation could be caused by two factors: (a) prolonged exposure to increasingly high salinities for a period of months, permitted physiological acclimatization, or (b) the increasing salinities obliterated the non-regulating crabs, thus selecting for strong regulators which were the only crabs remaining to be observed. Of course, both factors may be involved. In order to test these two possibilities a group of *Hemigrapsus* collected from Los Penasquitos lagoon when the salinity was in excess of 160% sea water was back-acclimated by gradual steps to 100% sea water. After a period of about two months in 100% sea water the crabs were immersed directly into 150% sea water for a period of 72 hours. The blood was then sampled and analyzed for total cations. Of seven surviving specimens thus treated only one demonstrated significant hypo-osmotic regulation. The blood of this one crab was equivalent in concentration to 134% sea water which is not as strong regulation as the weakest regulator observed in a

field situation where the lagoon water was 160% sea water (Fig. 1). Nevertheless, this is better regulation than has been reported for *Hemigrapsus* previous to the present investigation.

The results of this experiment are difficult to interpret because the loss of regulation may be a matter of physiological breakdown caused by the prolonged laboratory conditions. The experiment might be interpreted to mean that sudden transfer from normal to 150% sea water did not permit time for physiological acclimation which would occur in an isolated lagoon. Also, the fact that one specimen of this group was regulating in 150% sea water for a period of 72 hours, an ability not shown for *Hemigrapsus* either by Jones (1941) or Gross (1957a), suggests that some selection for hypo-regulation had taken place in the lagoon. Evidence was produced above that such selection had occurred in the case of *Pachygrapsus*. It seems reasonable that both selection and physiological acclimation were involved.

An attempt was made to acclimate *Hemigrapsus* gradually from 100% sea water to increasing salinities in order to demonstrate physiological acclimation, but the experiment failed because of a high mortality rate under the prolonged laboratory conditions.

Table I compares the blood ion concentrations of the two species of crab immersed in 100% sea water and 175% sea water. It should be pointed out that the

TABLE I

Ionic concentration in the blood of Hemigrapsus *and* Pachygrapsus

		100% sea water* (laboratory conditions)			175% sea water (lagoon field samples)		
		Mean	S.D.	No.	Mean	S.D.	No.
Na (meq/l.)	P	483	17.3	36	802	105	10
	H	452	10.3	6	726	39.3	10
	M	464			801		
K (meq/l.)	P	7.36	1.4	36	14.1	2.2	10
	H	8.13	1.1	6	12.5	1.9	10
	M	9.8			15.0		
Ca (meq/l.)	P	29.6	5.9	44	43.8	5.0	10
	H	37.7	5.3	5	51.0	10.3	10
	M	20.0			35.9		
Mg (meq/l.)	P	20.0	6.1	44	36.4	14.7	10
	H	70.0	13.3	5	135	31.4	10
	M	104			191		
Total (meq/l.)	P	540			896		
	H	568			925		
	M	598			1043		

P = *Pachygrapsus*
H = *Hemigrapsus*
M = Medium
* = Blood ion concentrations for *Pachygrapsus* in 100% sea water were reported previously (Gross, 1959).

values for crabs immersed in 175% sea water were determined from samples taken in the field. The values for normal sea water were for laboratory conditions for both species. However, the *Hemigrapsus* in this case were collected from Los Penasquitos lagoon returned to the laboratory and back-acclimated gradually to 100% sea water. Thus, the values for *Hemigrapsus* blood following immersion in normal sea water are subject to some question. Nevertheless, differences in the regulation of ions between *Pachygrapsus* and *Hemigrapsus* are salient in both salinities. In the first place, Na is regulated more strongly by *Hemigrapsus* than by *Pachygrapsus*. That is, the blood Na is significantly less concentrated in *Hemigrapsus* in both the normal and concentrated sea water than it is in *Pachygrapsus*, $P < 0.05$. On the other hand, blood Mg concentrations in *Hemigrapsus* are more than three times as high as they are in *Pachygrapsus* for both salinities. The regulation of K and Ca does not differ dramatically between the two species.

It is difficult to say on the basis of means that *Pachygrapsus* is the stronger hypo-osmotic regulator of the two species. Yet an examination of Figure 1 reveals that in the highest salinities where both species were studied, the strongest regulators, by far, were *Pachygrapsus* and the evidence is strong that *Pachygrapsus* survived *Hemigrapsus* at the extreme salinities. In view of these observations and the findings of earlier investigations (Jones, 1941; Gross, 1957a), it seems fair to consider *Pachygrapsus* the stronger regulating species in concentrated sea water. Yet it is suggested in Table I that the difference in osmo-regulatory ability is merely a difference in the ability to regulate Mg. That is, *Pachygrapsus* is a far stronger regulator of Mg than is *Hemigrapsus*.

Table II presents urine ion concentrations for the two crabs collected from 175% sea water. Since these samples were taken in the field, under conditions where contamination of urine samples with blood were more likely than under better controlled laboratory conditions, great precision cannot be placed on the concentration values. The mean urine Na for *Pachygrapsus* (572 meq/l.) is not significantly less than the mean urine Na for *Hemigrapsus* (687 meq/l.) although these concentrations would correlate well with the respective concentrations of Na in the blood of the two species. It is particularly interesting that the Mg concentration in the urine of the two species is about equal because as indicated above *Pachygrapsus* is a much stronger regulator of Mg than is *Hemigrapsus*. Values for urine Mg are smaller in *Pachygrapsus* than previously reported for lower salinities (Prosser *et al.*, 1955; Gross, 1959) which suggests that contamination with the blood had occurred. However, urine samples are more difficult to take from *Hemigrapsus* than *Pachygrapsus* and it is therefore likely that contamination of urine in *Hemigrapsus* also took place. It is concluded that while the values for urine Mg given in Table II are not precise, they strongly suggest that the antennary glands of both species are capable of concentrating Mg about equally.

Inspection of the total cation concentrations in urine and blood for both species (Table I and Table II) shows higher total ions in the urine than in the blood (meq/l.), but not knowing the anion constituents, this cannot be interpreted to mean that the urine is hypertonic to the blood. The blood and urine were compared by melting point determinations on six individual *Pachygrapsus* removed from the lagoon when the salinity was about 180% sea water. The average ratio, urine concentration/blood concentration, was 1.02 (range = 1.00 to 1.06). Thus, if the

TABLE II

Ionic concentration in field samples of urine taken from Hemigrapsus *and* Pachygrapsus *collected from 175% sea water*

		Mean	S.D.	No.
Na (meq/l.)	P	572	137	5
	H	687	84.8	8
K (meq/l.)	P	16.4	3.3	5
	H	12.0	1.9	8
Ca (meq/l.)	P	46.0	9.9	5
	H	56.9	14.9	8
Mg (meq/l.)	P	353	87.6	5
	H	364	195	8
Total (meq/l.)	P	987		
	H	1120		

P = *Pachygrapsus*
H = *Hemigrapsus*

urine is slightly more concentrated than the blood, it is not sufficiently so for the antennary glands to be effective as osmotic regulators.

Salinity preference was measured for *Pachygrapsus* collected from Los Penasquitos lagoon when the salinities were high at least six months after its isolation from the open sea. Nine crabs collected from the lagoon were individually placed in a salinity selectivity chamber for at least 36 hours where they could choose between 50%, 100%, 130% or lagoon water from which the particular crab had been collected (160%–180% sea water). A record was, therefore, obtained on the salinities visited and on the time spent in each. Adequate precautions were taken against choice for position rather than salinity. The procedure and apparatus are discussed by Gross (1957b). Thus, of the nine crabs, five preferred 100% sea water, two preferred 50% sea water, one preferred 130% sea water and only one demonstrated a preference for the salinity from which it had been collected. The mean time spent by all the crabs in the different salinities (% total time in the box) were: 50% sea water, 21.6; 100% sea water, 32.3; 130% sea water, 12.8 and lagoon water only 6.5% of the total time. It, therefore, seems likely that the preference for normal sea water previously described for *Pachygrapsus* persists even though the animal has been removed from normal sea water for months. It is even more apparent that it avoids the high salinities in which it had been living.

It has been reported by Gross and Marshall (1960) that the tissues of *Pachygrapsus* (as suggested by muscle) increase in water content when the animal is transferred from normal to dilute sea water and decrease in water content when the animal is transferred from normal to concentrated sea water, the resulting volume changes in the tissue taking place at the expense of the blood space. The above authors point out that such alterations in the anatomy of the blood space likely would affect the efficiency of the vascular system and consequently the adaptiveness of the animal. They suggested that volume control in the tissue might occur after

a prolonged exposure to osmotic stress, thus permitting the vascular system to return to a normal anatomy.

Ten *Pachygrapsus* collected from Los Penasquitos lagoon on July 12, 1960, were returned to the laboratory in Riverside where they were immersed in lagoon water (183% sea water) for an additional 24 hours. Samples of muscle tissue were removed from the legs of each crab, blotted uniformly, weighed, and dried to constant weight in a drying oven at 95° C. The difference between dry weight and wet weight was considered to be the water content of the tissue. Thus, the mean water content of samples from each of ten crabs was 69.24% wet weight, S.D. = 1.46. Water content of muscle from *Pachygrapsus* immersed in normal sea water was reported as 75.00% wet weight, S.D. = 1.40; from 150% sea water, 71.70% wet weight, S.D. = 1.71 (Gross and Marshall, 1960). All the above values are significantly different; $P < 0.01$. It, therefore, becomes apparent that the muscle of *Pachygrapsus* does not return to normal volume when immersed in concentrated sea water for a period of months and this is probably true for the other formed tissues. Thus, if the volume of the blood space is returned to normal after prolonged exposure to osmotic stress it must be brought about by other means. Burger and Smythe (1953) suggest that the nephridial bladders and stomach of *Homarus* serve such a purpose.

Discussion

The crabs *Pachygrapsus* and *Hemigrapsus* can be adapted to high salinities in two ways: (a) tolerance and (b) hypo-osmotic regulation. While the responses of *Pachygrapsus* to concentrated sea water in the field do not differ greatly from those reported for laboratory studies (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957a), the responses of *Hemigrapsus* are quite different from laboratory findings and point to a case where short-term studies have led to an erroneous conclusion, namely that *Hemigrapsus* cannot hypo-regulate.

Pachygrapsus might be considered the stronger hypo-regulator of the two species and as indicated above, this is likely made possible by its stronger ability to regulate Mg. It is particularly interesting, however, that *Hemigrapsus* should be a weaker regulator of Mg than *Pachygrapsus* because it seems equally capable of concentrating this ion in its urine (Table II).

Now a crab which is regulating in a concentrated medium tends to lose water with the physical gradient and also by way of the urine it excretes. Yet as previously demonstrated (Gross, 1957a) the crab (at least *Pachygrapsus*) retains its normal body volume when immersed in concentrated sea water. This means, of course, that an uptake of water must balance the loss. A highly permeable animal, losing relatively large volumes of water to the external medium by diffusion, could thus exclude only a small fraction of its water intake in the form of urine. Also, if water loss were compensated for by drinking, then a highly permeable animal, experiencing a large water loss, would drink relatively large volumes of the concentrated medium which would mean a large intake of Mg. There is evidence that the exoskeleton of *Hemigrapsus* is more permeable to water than that of *Pachygrapsus* (Gross, 1957a). It may be that *Pachygrapsus* because of its lower permeability can exclude a greater fraction of its water intake in the form of urine, than can *Hemigrapsus* which in turn would mean a greater exclusion of Mg. It might also

be that *Pachygrapsus* having a smaller water turn-over does not drink as much as does *Hemigrapsus* and consequently takes in less of the Mg-rich water.

It was indicated above that the crabs (particularly *Hemigrapsus*) of Los Penasquitos lagoon migrated out of the depths to the shores' edge when the salinities became high, yet it cannot be concluded that salinity *per se* caused this movement for there were other physical and biological factors involved. For example, the oxygen tensions in the depths of the lagoon were extremely low in the summer months and by the end of August, 1959, were nil (Carpelan, 1961). It is believed that the lack of oxygen was the most critical stress forcing the migration. There was some rotenone poisoning in limited areas of the lagoon on several occasions during the summer of 1959 (Carpelan, 1961). However, this is not believed to have influenced the migration because the poison is a highly unstable substance which could be effective only for a short period in the limited areas where used. Besides, rotenone was used in early June and July, yet *Hemigrapsus* did not move into the shallows until August. Surface temperatures for several locations in the lagoon remained between 25° and 29° C. during July, August and September of 1959 (Carpelan, 1961). Thus, it does not seem that temperature could be critical in forcing the shoreward migration.

Los Penasquitos lagoon presents the physical stress which would tend to select for hypo-osmotic regulation, that is, high salinities. The lagoon also becomes stagnant, and in acquiring anaerobic conditions presents a stress which could force animals capable of movement toward the shore and out of the water. Thus, a selective pressure favoring aerial respiration is presented by the lagoon. While there are no available records of the salinities of this lagoon during the rainy season of a wet year, Carpelan (1961) has shown significant decreases in salinity following the light rains of 1959 and it is likely that run-off during heavy rains could dilute the lagoon water considerably.

If dilute as well as hypersaline conditions in coastal lagoons of the past were common, then the correlation between osmotic regulation and terrestrial life among crabs can be explained as a reflection of the history of the animals rather than necessarily a physiological adaptation to habitats in which the animals are typically found today. The adaptive significance of osmotic regulation in semi-terrestrial and terrestrial crabs was questioned by Gross (1955).

It is suggested, therefore, that coastal lagoons might have afforded ideal conditions for the evolution of terrestrial crabs. That is, selective pressures are available in these bodies of water favoring: (1) hyper-osmotic regulation, (2) hypo-osmotic regulation, and (3) aerial respiration, three physiological conditions common to crabs showing degrees of the terrestrial habit.

It probably would have been necessary for the crabs to be trapped as were the Los Penasquitos populations so that obligatory selection would have been imposed upon them. Also it probably would have been necessary that access to the open sea and normal salinities be seasonally available. Inasmuch as ovigerous crabs were not found in Los Penasquitos lagoon when the salinities were high, it is unlikely that reproduction occurred in the lagoon during the stressed conditions. However, this does not mean that such a population would be non-breeding, for if the isolated body of water were opened to the sea periodically, then normal salinities would become available which would permit reproduction and the escape

of free-swimming larvae to the open sea. It may be that the retention of a preference for normal sea water in *Pachygrapsus* after months in hypersalinities is an adaptation which assures reproduction in normal salinities. The periodic availability of normal sea water might explain why the land crabs have not evolved reproductive mechanisms for terrestrial life.

It does not follow that lagoons would have had to dominate the coastal areas where land crabs evolved, although lagoons which are opened intermittently to the sea during high equinoctical tides and rainy seasons are common today on the coast of South Africa, Brazil, Australia and south Texas (Ladd *et al.*, 1957). Neither would it be necessary for the majority of a particular species to inhabit the lagoons. All that would be required would be large absolute populations within the lagoons where they would be rigorously selected for the three physiological features which would have to have hereditary components. It also would be required that the three physiological factors would not be selected against in normal sea water. Thus, adults which were severely selected in an isolated lagoon for the ability to regulate osmotically and to respire in the air would periodically reproduce. Their young would then escape to the open sea and in effect increase the gene frequency for the characters in question within the general population. Given sufficient time, the characters could be common to the species. Of course, once the first step was made toward terrestrial life selective pressures might favor this feature in situations outside of the lagoon.

Edney (1960) emphasizes that the evolutionary route toward land directly across the littoral zone from the sea is more difficult than by way of estuaries and swamps because the problems of terrestrial adaptation are imposed at once. Gislén (1947) considers estuarine mangrove swamps as most likely pathways toward terrestrial life. Hedgepeth (1957) discusses the open marine beach as a direct route for landward migration. Neither of these possibilities, however, explains the common occurrence of both hyper- and hypo-osmotic regulation among terrestrial crabs.

On the other hand, conditions in coastal lagoons such as described above could not only provide the selective pressures favoring both types of osmo-regulation, but also could provide the conditions whereby the initial step from sea to land could be evolved in a gradual, but obligatory manner.

The direct invasion of land across a marine beach would require an initial step where the stresses of aerial life would be imposed suddenly. It would seem that the disadvantage of such a sudden step would outweigh the advantages of becoming land-borne. Perhaps crabs which appear to have become land-borne directly across the littoral zone (*e.g. Ocypode*) have in their history experienced selective pressures favoring such a move in ecological situations (lagoons of varying salinity) which differ from their present typical habitats.

Ferronnière (1901) was one of the earlier workers to point out that animals capable of tolerating low salinities commonly are also capable of tolerating high salinities. He also observed that it is common for species of the supra-littoral zone to inhabit brackish as well as hypersaline waters.

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Summary

1. The crabs *Pachygrapsus crassipes* and *Hemigrapsus oregonsis* were found thriving in an hypersaline lagoon which had been isolated from the sea for months and had attained salinities in excess of 175% of normal.

2. Contrary to previous reports *Hemigrapsus* was found to be a strong hypo-osmotic regulator in concentrations as high as 175% sea water. The discrepancy between previous laboratory findings and the field results of the present investigation are attributed to the prolonged acclimatization period in lagoon water of gradually increasing salinity and to severe selection permitting only hypo-regulators to be sampled.

3. No living *Hemigrapsus* were found in the lagoon when the salinities had exceeded 180% sea water.

4. *Pachygrapsus,* as was expected from previous studies, was regulating osmotically in the lagoon when the concentrations were above 185% sea water.

5. Both *Pachygrapsus* and *Hemigrapsus* can tolerate blood concentrations in excess of 160% sea water and remain active.

6. *Pachygrapsus* is a stronger hypo-osmotic regulator than *Hemigrapsus.* Since it maintains its blood Mg considerably lower than does *Hemigrapsus* in concentrated sea water, the stronger ability to hypo-regulate may be made possible by the greater capacity on the part of *Pachygrapsus* to excrete Mg.

7. The urine Mg for *Hemigrapsus* is about equal in concentration to that of *Pachygrapsus* when the crabs are immersed in 175% sea water, even though the blood Mg concentration is more than three times higher than that of *Pachygrapsus.*

8. *Hemigrapsus* is a stronger regulator of Na in hypersaline water than is *Pachygrapsus.*

9. The antennary glands in both *Pachygrapsus* and *Hemigrapsus* are ineffective as organs of hypo-osmotic regulation.

10. When *Pachygrapsus* is transferred from normal to concentrated sea water the volume of its muscle tissue, as indicated by water content, becomes reduced. This reduction in volume persists in hypersaline waters even after months of immersion. Thus, there is no evidence of volume control for muscle tissue.

11. After months of isolation in a hypersaline lagoon *Pachygrapsus* shows a preference for normal sea water when offered a choice of salinities and will avoid the hypersaline water from which it has been captured.

12. Evidence is produced showing that coastal lagoons present selective pressures favoring: (1) hyper-osmotic regulation, (2) hypo-osmotic regulation, and (3) aerial respiration, three physiological characteristics common to terrestrial and semi-terrestrial crabs. Coastal lagoons are suggested as ideal sites for the evolution of land crabs.

Literature Cited

Burger, J. W., and C. Smythe, 1953. The general form of circulation in the lobster *Homarus.* *J. Cell. Comp. Physiol.,* **42**: 369–383.

CARPELAN, L., 1961. Salinity tolerances of some fishes of a Southern California coastal lagoon. *Copeia,* **1961**: 32–39.

EDNEY, E. B., 1960. Terrestrial adaptations. *In:* The Physiology of Crustacea (Waterman, Ed.), Vol. 1, Chapter 11: 367–393. Academic Press, New York and London.

FERRONNIÈRE, G., 1901. Études biologiques sur les zones supralittorales de la Loire-Inférieure. *Bulletin de la Société des Sciences Naturelles de L'Ouest de La France. Series 2,* **1**: 1–451.

GILSÉN, T., 1947. Conquering terra firma. *Proceedings of the Royal Physiographic Society, Lund,* **17**: 216–235.

GROSS, W. J., 1954. Osmotic responses in the sipunculid *Dendrostomum zostericolum. J. Exp. Biol.,* **27**: 96–109.

GROSS, W. J., 1955. Aspects of osmotic regulation in crabs showing the terrestrial habit. *Amer. Nat.,* **89**: 205–222.

GROSS, W. J., 1957a. An analysis of response to osmotic stress in selected decapod Crustacea. *Biol. Bull.,* **112**: 43–62.

GROSS, W. J., 1957b. A behavioral mechanism for osmotic regulation in a semi-terrestrial crab. *Biol. Bull.,* **113**: 268–274.

GROSS, W. J., 1959. The effect of osmotic stress on the ionic exchange of a shore crab. *Biol. Bull.,* **116**: 248–257.

GROSS, W. J., AND L. A. MARSHALL, 1960. The influence of salinity on the magnesium and water fluxes of a crab. *Biol. Bull.,* **119**: 440–453.

HEDGEPETH, J. W., 1957. Sandy beaches. *In:* Treatise on Marine Ecology and Paleoecology. Vol. 1, Chapter 19: 587–608. Geological Society of America, Memoir 67.

JONES, L. L., 1941. Osmotic regulation in several crabs of the Pacific Coast of North America. *J. Cell. Comp. Physiol.,* **18**: 79–91.

LADD, H. S., J. W. HEDGEPETH AND R. POST, 1957. Environments and facies of existing bays on the central Texas coast. *In:* Treatise on Marine Ecology and Paleoecology, Vol. 2, Chapter 22: 599–639. Geological Society of America, Memoir 67.

PROSSER, C. L., J. W. GREEN AND T. J. CHOW, 1955. Ionic and osmotic concentrations in blood and urine of *Pachygrapsus crassipes* acclimated to different salinities. *Biol. Bull.,* **109**: 99–107.

SOME EVOLUTIONARY PATTERNS IN FISHES' BLOOD[1]

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Electrophoretic analyses of serum protein fractions of some fishes and certain other animals have been presented by Deutsch and Goodloe (1945), Deutsch and McShan (1949), Engle *et al.* (1958), Irisawa and Irisawa (1954), and Moore (1945). In general it was shown that there are characteristic, reproducible, species differences in the plasma proteins and differences between the lower and higher animals. Deutsch and McShan give data on the blood plasmas of 16 fresh-water fishes and Engle *et al.* gave data on 20 species of marine fishes. Both groups of authors reported that gamma globulin fractions in the plasma of teleost fishes were absent or diminished, but Engle *et al.* found them high in the elasmobranchs.

Sulya *et al.* (1961) presented plasma protein data derived by paper electrophoresis from 183 specimens of 26 species of fishes from the Gulf of Mexico, and this is the most extensive series of data on fishes presented so far. In that report the techniques are described, with a discussion of certain physiological and clinical implications.[2] Gamma globulin was found in all teleosts and in some it was quite high. Data were given by species and there was some discussion of differences between the various fishes.

We propose here to call attention to certain facts of evolutionary significance, derived from Sulya *et al.* (1960 and 1961), and to present part of the unpublished data.

The Data

The electrophoretic patterns were illustrated by Sulya *et al.* (1961), but the tables, which list the plasma protein components of each species, were too long for publication. Therefore, those data have been deposited as Document 6454 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. They may be purchased as photoprints or on 35-mm. film by advance payment of $1.25 by check or money order made out to Chief, Photoduplication Service, Library of Congress.

The data presented in Table I are average values for the various Orders of fishes, arranged in a series from the generalized to the more specialized, according to the classification of Berg (1947). In general this arrangement conforms to the sequence from lower to higher fishes, as presently understood. The original data were the mean values for all specimens of the given species. The species were then

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[2] Sulya *et al.* (1961) have been criticized for not offering full information regarding quantification, especially staining of the strips. For that reason we wish to state here, somewhat out of context, that the procedure used was Spinco "Procedure A" in which the dye is bromphenol blue.

TABLE I

Plasma protein distribution in fishes arranged in the order of increasing evolutionary development

Order	Number of species	Total protein gms. %	Albumin gms. %	Globulins gms. %				Other plasma globulins
				α_1	α_2	β_1	γ_1	
Lamniformes (sharks)	3 (4)*	2.18			.695	.805	.539	$.143\gamma_2$
Lepidosteiformes (gars)	3 (5)*	2.87		.125	.344	1.386	1.011	
Clupeiformes (herring-like fishes)	4 (63)*	3.59	.082	.988	1.144	.810	.421	$.122\gamma_2$
Siluroidea (catfishes)	2 (10)*	2.94	.688	.226	.429	.546	1.159	
Mugiliformes (mullets)	2 (23)*	3.78	.550	.893	1.127	.378	.811	
Perciformes (perch-like fishes)	12 (78)*	3.70	.291	.661	.889	.723	.658	$.048\alpha_1(2)$ $.108\alpha_2(2)$ $.041\beta_2$ $.230\gamma_2$

* The number in parenthesis represents the number of animals.

all given equal rank, regardless of the numbers of specimens, and the means for the species values are presented as the values for the Orders in the table. Thus 12 values were averaged for the Perciformes and only two for the Mugiliformes.

The writers had hoped to utilize the data on fish plasma previously published, along with our own, so as to have a more extensive series of fishes in the tables. However, previous studies were carried out by the moving boundary electrophoresis while our own were made by paper electrophoretic methods, and there is a basic difference between the patterns obtained by the two methods. In the moving boundary procedure, the pattern is obtained from the refractive index gradient relative to cell level and the pattern represents, therefore, all refracting components irrespective of their chemical composition. On the other hand, the paper electrophoresis method measures only those components which combine with the staining dye. The data obtained by the two methods may be similar, in general, but not necessarily so. In some instances we observed fish plasma cholesterol values that were very high relative to higher vertebrates. In these cases the lipids may be expected to contribute to refraction, thus giving the associated fractions higher values in the moving boundary procedure.

DISCUSSION

Of the four major groups of living fishes (Elasmobranchii, Holocephali, Dipnoi and Teleostomi) we have representatives only of the first and the last.

However, these two Classes are widespread and numerous (which is the reason we have specimens), and the other two are somewhat side-issues to the main stem of evolution in fishes. It should also be recalled that the Teleostomi are divided into two subclasses, the Crossopterygii and the Actinopterygii, the first being represented among living fishes only by *Latimeria,* of South Africa and Madagascar, and we have no specimens. The main line of evolution of the higher vertebrates probably passed through the Crossopterygii, but this group is an evolutionary side issue in fishes and in fact it is virtually extinct. Therefore, the data presented cover representatives along the main stem of evolution in fishes, as represented by living species, somewhat better than might appear at first glance. One large gap concerns the most primitive of the Actinopterygii, the sturgeons and paddlefishes.

The table shows that there is gradual increase in the percentage of total plasma protein from lower to higher fishes. Only the Clupeiformes are out of sequence. In high protein content of the plasma the Clupeiformes are similar to the advanced rather than to the lower fishes. This Order corresponds largely to the Isospondylii of Jordan (1923) and previous workers. The Perciformes (Acanthopterygii, of Jordan and others), the most specialized fishes, have about a 70% higher content of plasma proteins than the elasmobranchs. There is considerable variation between the species and among the Perciformes the range was 1.68 to 6.19 grams of plasma proteins per 100 ml. of plasma. The plasma protein content of fishes is low as compared to mammals.

Irisawa and Irisawa (1954) first showed that albumin was not present in the sera of two elasmobranchs, a skate and a shark. Drilhon and Fine (1959) made similar observations on two species of sharks, both by electrophoretic and reagent methods. Sulya *et al.* (1961) found the same condition in three other species of sharks. Additionally, Table I shows that there was no albumin in the plasma of three species of gars. These are fresh-water fishes which sometimes enter brackish waters, where all of our specimens were caught. Furthermore, there was no albumin in the plasma of two (*Dorosoma cepedianum* and *D. petenense*) of the four clupeid fishes examined. Two of the twelve species of perciform fishes examined (*Cynoscion arenarius* and *Pogonias cromis*) also lacked albumin in the blood plasma. Analbuminemia seems to be the common condition among the lower fishes; it gradually becomes less prevalent as the fishes become more specialized but does not entirely disappear, and a small per cent of the higher fishes lack albumin in the blood plasma. Thus analbuminemia among fishes seems to be a primitive characteristic. However, Deutsch and McShan (1949) reported albumin in the plasma of sturgeons, the most primitive of living Teleostomi, and that group merits further attention.

The table shows that the sharks, gars, catfishes and mullet had four globulin components in the plasma, and the clupeids have five. It is clear that the perciform fishes, with eight types of globulin, have generally more complex plasma proteins than the lower groups.

Species data show that ten of the perciform fishes had four or five plasma globulin fractions. The croaker, *Micropogon undulatus,* had only two fractions, but the speckled squeteague, *Cynoscion nebulosus,* had eight. These two fishes are considered by most authorities to be in different sub-families of the family

Sciaenidae. One of the gars and one of the sharks were found to have only two globulin fractions; the other two species in each group contained three and four fractions.

In terms of average numbers of globulin fractions per species for each Order, there is an increasing complexity up the evolutionary scale, but again the Clupeiformes are out of sequence. The mean numbers of globulin fractions per species are: sharks 3, gars 3, clupeids 4.5, catfishes 3.5, mullets 4, percoids 4.5.

Species differences are clearly reflected in the plasma protein complexes. For instance, *Dorosoma petenense* possesses gamma-2 globulin, but *D. cepedianum* does not, and the latter has over 11 times as much alpha-1 globulin as the former; one of the catfishes has alpha-1 globulin, but the other does not; the striped mullet has ten times as much albumin as the silver mullet and only one-fourth as much gamma-1 globulin. On the other hand, the white squeteague, *Cynoscion arenarius,* and the black drum, *Pogonias cromis,* have remarkably similar plasma proteins.

Berg (1947) separated the Mugiliformes from the Perciformes because they have abdominal pelvic fins, which is a primitive characteristic. In general the blood plasma proteins of the mullet are less complex than in the Perciformes and similar to certain species of the catfishes and clupeids. This gives some corroborative evidence to the correctness of Berg's separation of the Mugiliformes from the Percomorphi. On the other hand, the plasma proteins of the mullets are also similar to certain perciform species of the families Carangidae, Sciaenidae and Scombridae. Thus, the difference between the plasma proteins of lower and higher fishes is a general character of the groups which does not always hold true when certain species are compared.

The Clupeiformes studied here have a higher plasma protein content and greater protein complexity than would be expected from their position on the evolutionary scale. On the other hand, they show primitive affinities in that half of the four species examined possess no albumin, and the other two only have small amounts.

Sulya *et al.* (1960) confirmed previous reports on the high electrolyte content in the blood of marine elasmobranchs. They found that the three major electrolytes, Na, K and Cl, were about 66% higher in the plasma of sharks than in the teleosts. They also found that the total cholesterol, including cholesterol esters, in plasma of sharks and gars is comparable to the levels in higher vertebrates, 173–151 mg.%, respectively, but in all the remaining teleosts it was considerably higher (Clupeiformes 663, Siluroidea 367, Mugiliformes 560 and Perciformes 606). These are mean figures for the Orders and species variations are averaged out. It was pointed out that there seems to be an association of plasma cholesterol content and concentrations of certain plasma proteins. Resemblances of the normal blood in fishes to the blood of the nephrotic rat and of man with biliary cirrhosis were noted. It appears that the normal condition in fishes' blood is comparable in some ways to certain pathological conditions of mammals, including man. This point is significant, not only from the evolutionary standpoint, but as indicating a new line of approach to the understanding of certain types of pathology of the kidney and liver of mammals. In this regard, it should be noted that Baril *et al.* (1961) called attention to resemblances of the serum of young alligators to that of humans with renal disease.

SUMMARY

1. A study was made, by electrophoretic techniques, of the plasma proteins of 26 species of elasmobranch and teleostome fishes from the northern Gulf of Mexico.

2. A general increase was found in the amount of plasma proteins from lower to more specialized fishes. Elasmobranchs lacked albumin in the plasma. This condition of analbuminemia also held true for all the gars and half of the Clupeidae studied; it generally disappeared in the more specialized fishes, but not entirely, and was found in two species of the Perciformes. There was a gradual increase in the number of globulin fractions of the plasma, from lower to higher fishes, among the Orders of fishes, which did not always hold true at species levels.

3. Closely related species could be distinguished by the presence or absence and varying amounts of plasma proteins, but there were exceptions and certain species in the same family could only be differentiated doubtfully on that basis.

4. The plasma proteins of the fishes examined showed a trend of increasing complexity from the generalized to the more specialized fishes.

5. The content of the chief electrolytes, Na, K and Cl, in the plasma of marine sharks was found to be much higher than in teleosts, in confirmation of earlier workers.

6. Total cholesterol in the plasma of sharks and gars was found in the same range as in mammals, but in the remaining teleost fishes it was a great deal higher.

7. Relations between cholesterol and plasma proteins in the blood of fishes resemble pathological conditions in mammals, including man.

LITERATURE CITED

BARIL, E. F., J. L. PALMER AND A. H. BARTEL, 1961. Electrophoretic analysis of young alligator serum. *Science*, **133**: 278–279.

BERG, L. S., 1947. Classification of Fishes both Recent and Fossil. 436 pp. J. W. Edwards. Ann Arbor, Michigan.

DEUTSCH, H. F., AND M. B. GOODLOE, 1945. An electrophoretic survey of various animal plasmas. *J. Biol. Chem.*, **161**: 1–20.

DEUTSCH, H. F., AND W. H. MCSHAN, 1949. Biophysical studies of blood plasma proteins. XII. Electrophoretic studies of blood serum proteins of some lower animals. *J. Biol. Chem.*, **180**: 219–233.

DRILHON, ANDRÉE, AND JEAN-M. FINE, 1959. Les protéines du serum sanguin chez les Elasmobranches (*Scyllium catulus* et *Scyllorhinus canicula*). *C. R. Acad. Sci.*, **248**: 2418–2420.

ENGLE, R. L., K. R. WOODS, ELIZABETH C. PAULSEN AND J. H. PERT, 1958. Plasma cells and serum proteins in marine fish. *Proc. Soc. Exp. Biol. and Med.*, **98**: 905–909.

IRISAWA, H., AND A. F. IRISAWA, 1954. Blood serum protein of the marine Elasmobranchii. *Science*, **120**: 849–851.

JORDAN, D. S., 1923. A classification of fishes, including families and genera as far as known. *Stanford Univ. Publ., Biol. Sci.*, **3**: 77–243.

MOORE, D. H., 1945. Species differences in serum protein patterns. *J. Biol. Chem.*, **161**: 21–32.

SULYA, L. L., B. E. BOX AND G. GUNTER, 1960. Distribution of some blood constituents in fishes from the Gulf of Mexico. *Amer. J. Physiol.*, **199**: 1177–1180.

SULYA, L. L., B. E. BOX AND G. GUNTER, 1961. Plasma proteins in the blood of fishes from the Gulf of Mexico. *Amer. J. Physiol.*, **200**: 152–154.

THE DISTRIBUTION OF MINERAL MATERIAL IN THE CALCIFIED CARAPACE AND CLAW SHELL OF THE AMERICAN LOBSTER, HOMARUS AMERICANUS, EVALUATED BY MEANS OF MICROROENTGENOGRAMS [1, 2]

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The histology of the calcified crustacean integument has been investigated by means of the light microscope (Williamson, 1860; Vitzou, 1882; Herrick, 1895; Drach, 1939; Dennell, 1947; Travis, 1955, 1957, 1960) and the polarizing microscope (Prenant, 1927a, 1927b; Dudich, 1931; Drach, 1939). Some aspects of calcification in relation to shell structure have been reviewed by Richards (1951).

The calcium carbonate found in the exoskeleton of the European lobster, *Homarus gammarus,* exists in an amorphous form (Prenant, 1927a). X-ray diffraction patterns obtained from the dry, intact carapace of the American lobster indicate that some calcite is present, but that much of the mineral does not have a distinct crystalline structure (Glimcher, personal communication).

Microroentgenography has been a useful technique in the evaluation of the degree of mineralization in various types of calcified tissue (Engström *et al.,* 1957). This report discusses microroentgenograms of lobster shell which were obtained using comparatively thick sections of calcified integument from the carapace and the claw shell. By exposing the animals to sea water labelled with calcium-45 for a short period of time immediately before sacrifice, labile areas of mineral could be identified.

Materials and Methods

Two male lobsters, 1 (518 grams) and 2 (436 grams), were obtained live from a local market. The lobsters were in intermolt condition, stage C-4 (Drach, 1939). The animals were allowed to remain in a tank of artificial sea water for seven days. They were then placed for two hours in an artificial sea water solution labelled with calcium-45 and strontium-85 which contained 25 mEq./l. calcium. After a two-hour period, the lobsters were removed from the labelled sea water, rinsed

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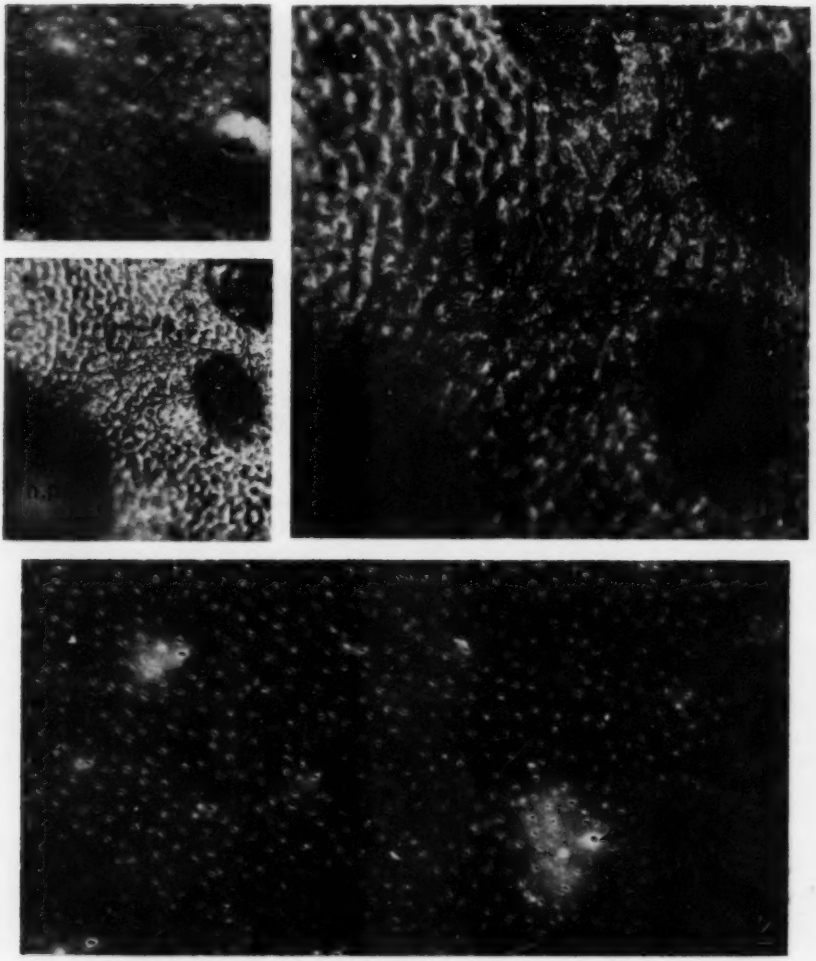


FIGURE 1. Surface view of dorsal claw shell, above muscle attachment.
FIGURE 1a. Obliquely reflected light. 30 × magnification.
FIGURE 1b. Directly reflected light. 250 × magnification.
FIGURE 1c. Directly reflected light. 500 × magnification.
FIGURE 2. Planar section, claw 1, principal calcified zone. Microroentgenogram. Enlarged 42 ×. h.p. = hair pore, t.d. = tegumental gland duct, p.c. = pore canal.

in a bath of distilled water, and blotted with gauze sponges. The posterior half of the carapace and the flat portion of the claw above the point of muscle attachment were removed. Pieces of shell about 20 mm.^{2} were obtained from the flat dorsal surface of the large member of the crusher claw above the point of muscle

attachment and 15 mm. from the tip of the claw. Carapace shell samples of similar size were also obtained at a point 15 mm. from the posterior and from the lateral borders of this tissue. The shells were rinsed briefly with a second jet of distilled water, and the non-calcified membrane was partially stripped off. Shells were dried at 100° C. for 12 hours.

A randomly selected area of dorsal claw shell, above the point of muscle attachment, was photographed at a magnification of 30 × by obliquely reflected light (Fig. 1a). By means of a metallurgical microscope, photographs of the shell by directly reflected light were obtained (Figs. 1 b-c). Dr. M. E. Nicholson of the Metallurgy Department of this University assisted with this procedure.

Irregular pieces of shell, about 16 mm.^{2}, were embedded in methyl methacrylate. One hundred-micron longitudinal and transverse sections were cut as well as one transverse 50-micron section from the carapace. The sections were polished on both sides with emery paper and placed directly upon the emulsion side of Kodak Maximum Resolution plates. Microroentgenograms were prepared according to the technique described by Bergman and Engfeldt (1954) and Engström *et al.* (1957). Target-to-film distance was at least 25 cm. Polychromatic radiation from a Machlett x-ray tube, with a tungsten target and a beryllium filter, with 6 KV tension, were used to obtain the microroentgenograms. The developed microroentgenograms were photographically enlarged. Positive prints of these enlargements are shown in Figures 2–8. In these figures, areas which are white in the microroentgenogram print represent areas of greater mineral concentration than areas with a gray cast.

To obtain photomicrographs of the claw sections, a reflected light source was used, while a transmitted light source was employed in the preparation of the photomicrographs of carapace sections.

Autoradiographs were prepared by placing the tissue sections in contact with the emulsion side of Kodak glass lantern slide plates. The tissues were allowed to remain in contact with the glass plates four months before the plates were developed. Darkening of the photographic film was shown to be due to beta particles from calcium-45 rather than gamma rays from strontium-85. Aliquots of solutions containing either calcium-45 or strontium-85 were pipetted onto blotter paper planchettes, designed so that a planchette of approximately the same size as a shell sample contained approximately the same amount of activity. After the planchettes were dry, they were placed in contact with the photographic plates for two months. No darkening due to strontium-85 gamma rays was observed when the plates were developed after this time.

The calcium content of adjacent fragments of shell was determined by the method of Clark and Collip (1925) after the shells had been ashed at 450–500° C. The calcium concentrations, expressed in mEq./g. dry shell, were as follows:

Carapace	1:	13.1 mEq./g.,	2:	13.8 mEq./g.
Claw	1:	11.5 mEq./g.,	2:	12.7 mEq./g.

The total residue remaining after ashing was as follows, expressed as per cent of dry weight:

Carapace	1:	67.7%	2:	70.1%
Claw	1:	60.5%	2:	66.6%

Description of photomicrographs, microroentgenograms, and radioautographs

Figure 1. Surface view of claw shell, randomly selected from dorsal surface of claw, above point of muscle attachment.

In Figure 1a, the openings of the hair pores (h.p.) and of the tegumental gland ducts (t.d.) are visible at 30 times magnification. In Figures 1b and 1c, 250 × and 500 × magnification, one hair pore is visible in the lower left hand corner. The tegumental gland duct openings (t.d.) are visible as large, circular structures. The many fine circular structures (p.c.) probably represent the points at which the pore canals initially opened to the surface. These figures were included to demonstrate the fact that there are three main types of openings or potential openings on the surface of the claw.

Figure 2 is a microroentgenogram, enlarged 42 times, of a planar section of the claw through the principal calcified zone (lobster 1). The microroentgenogram indicates that there are two circular or oval types of structure around which the density of the mineral is increased. Carlström (personal communication) has suggested that these could be areas in which the calcium carbonate possesses a crystalline structure. The larger dark spots (h.p.) surrounded by a bright halo probably represent transverse cuts through the pores of setae. They occur with about the same frequency as do the large pores identified as hair pores in Figure 1a. The smaller dark spots (t.d.) surrounded by areas of increased mineralization are the ducts of tegumental glands. The external openings of these ducts may be seen in Figures 1a-c. The slightly elliptical appearance of some of the passages in this microroentgenogram is due to the fact that the shell is curved and some of the pores have been cut at an angle greater or less than 90°.

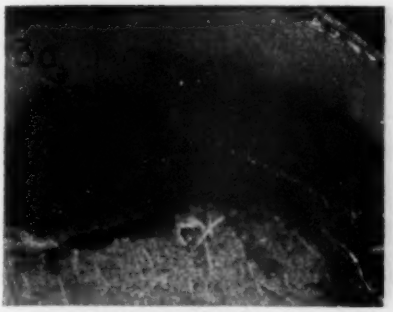
Figure 3a is a photomicrograph of a section which passes diagonally through the pigmented layer of the claw of lobster 2. The light triangular area on the right is methyl methacrylate as is the light area at the base of Figure 3a. The dark area in the center of the figure is the upper, heavily pigmented portion of the layer, and the lighter area immediately above represents the less heavily pigmented lower portion of this layer. The shell sample cracked when it was cut and polished and the break can be seen passing diagonally across the section.

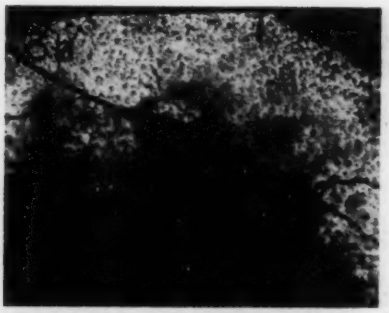
The microroentgenogram, Figure 3b, of this section of the claw indicates that there is slightly more mineralization in the outer strata than in the inner strata of this layer. Areas of lesser calcification probably represent ducts and pores or centers of cuticular prisms.

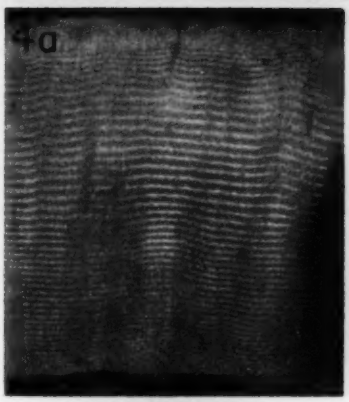
From the radioautograph of this section, Figure 3c, it is difficult to observe differential uptake of isotope in this layer. It appears that the lower and middle portions of the layer contain slightly more activity than the outermost portion.

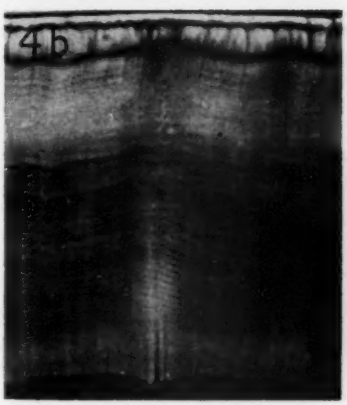
Figure 4a represents the photomicrograph of a transverse section of claw (Lobster 1). The division between the pigmented layer and the principal calcified zone can be observed. There is some indication of the possible initial location of the interprismatic septa in the pigmented layer, although in *Homarus* the septa actually fuse at an early stage after the molt is completed (Yonge, 1932; Dennell, 1960).

In the microroentgenogram of this section of claw, Figure 4b, a thin layer of greater mineralization can be observed on the outer surface of the shell. This area probably corresponds to the region of the lower epicuticle and the upper pigmented layer. Drach (1939) stated that in the Brachyura, calcite crystals









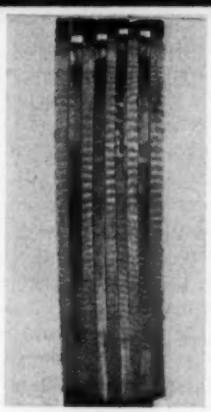


FIGURE 3. Planar section, claw 2, pigmented layer. Enlarged 48 ×.
FIGURE 3b. Microroentgenogram.
FIGURE 3c. Radioautograph.
FIGURE 4. Transverse section, claw 1. Enlarged 66 ×.
FIGURE 4a. Photomicrograph, reflected light.
FIGURE 4b. Microroentgenogram.
FIGURE 4c. Alternation of strips from microroentgenogram and radioautograph.

overlap between the two layers. The stratified nature of the pigmented layer, mentioned by Vitzou (1882), Herrick (1895), and Dennell (1960), is obscured by vertical "columns" of mineral material. The "columns" are wider at the outer surface. Dennell (1960) states that the interprismatic spaces are sites of early mineralization. From these sites, mineralization proceeds laterally and downward. It is possible that the areas which are less well mineralized represent interior portions of the cuticular prisms.

The strata of the principal calcified zone, underlying the pigmented layer, are clearly defined. The density of mineralization is actually less than that observed in the upper third of the pigmented layer. The most noticeable features of the principal calcified zone are the alternation in the density of the mineral matter in the horizontal strata and the two types of observed "ducts" which pass through the zone. The most numerous, clearly observed at the base of the principal zone, are probably the tegumental gland ducts. The large "duct" at the middle base of the principal zone is either an extremely large tegumental gland duct or the hair pore of a seta.

Figure 4c represents an attempt to correlate the strata of the principal calcified zone which may be observed in the microroentgenograms with those seen in the photomicrographs. Strips ⅛ inch wide were cut, beginning at the same location, from both photomicrograph and microroentgenogram. The section was then reconstructed, using alternate strips from the two photographs. To some extent, the dark striations of the photomicrograph correspond to the dark striations of the microroentgenogram. The correlation is not perfect, and this can be regarded as suggestive only.

Figures 5a and 5b show the microroentgenogram and radioautograph obtained from transverse section from the claw of lobster 2. Details observed in 5a are similar to those seen in Figure 4b. There appears to be a greater concentration of the radioisotope in the outer layers of the claw.

In Figure 6a, a transverse section of the carapace from lobster 2 is shown. This photomicrograph, obtained with transmitted light, merely indicates differences between outer and inner portions of the pigmented layer and the location of the principal calcified zone. The horizontal striations in the principal calcified zone, which can be easily observed at a broken edge of shell, are difficult to observe in this photograph. The very dark vertical line is an artifact.

In the microroentgenogram of this section, Figure 6b, the mineral appears to be more uniformly distributed in the principal calcified zone. This may be due to greater density of the carapace shell as compared to the claw shell. In the radioautograph of the section, Figure 6c, calcium-45 activity is distributed in a much less uniform manner than in claw sections. There is little apparent relationship between uptake of isotope and variation in mineralization.

Figure 7 is a microroentgenogram of a carapace section, Lobster 1, half the thickness of that used to obtain the photographs in Figures 6a-b. There is more detail visible in the pigmented layer. However, strata in the principal calcified zone are only faintly visible, indicating that mineral is indeed more uniformly distributed in this zone in the carapace than in the claw. The duct observed is probably the hair pore of a seta.

Figures 8a, b, and c were obtained using a planar section of carapace (lobster 2)

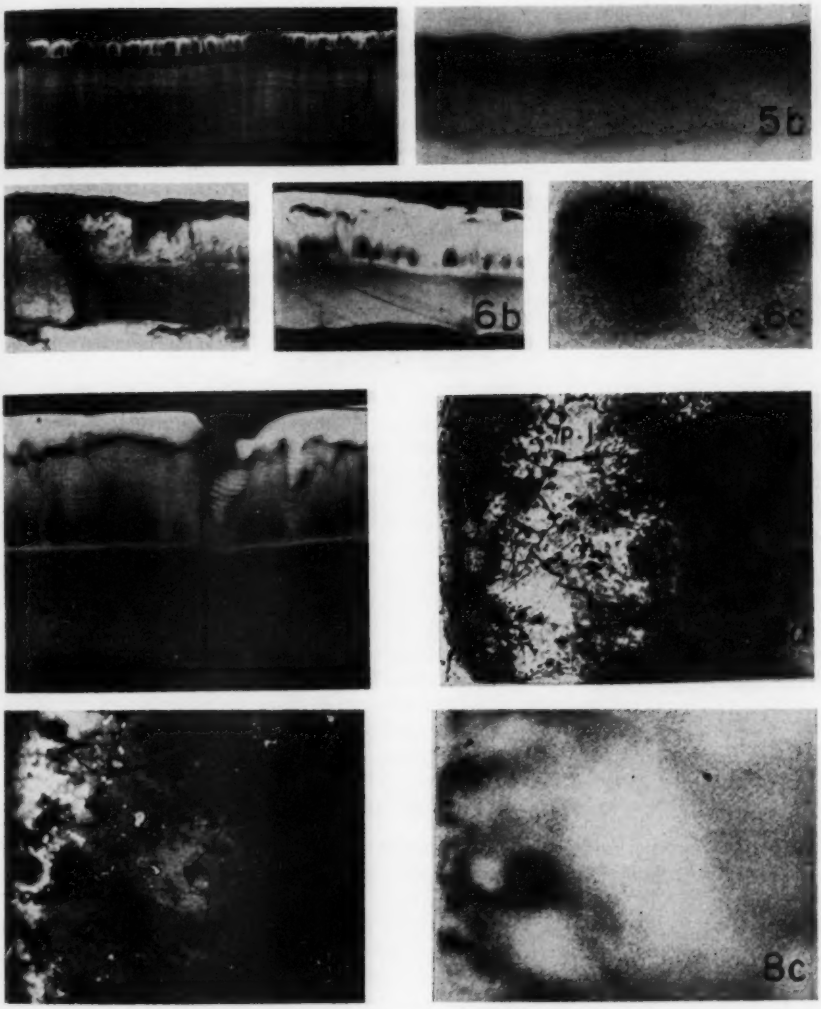


FIGURE 5. Transverse section, claw 2. Enlarged 22×.
FIGURE 5a. Microroentgenogram.
FIGURE 5b. Radioautograph.
FIGURE 6. Transverse section, carapace 2. Enlarged 66×.
FIGURE 6a. Photomicrograph, transmitted light.
FIGURE 6b. Microroentgenogram.
FIGURE 6c. Radioautograph.
FIGURE 7. Transverse section, carapace 1. Enlarged 98×. Microroentgenogram.
FIGURE 8. Planar section, carapace 2. Enlarged 60×.
FIGURE 8a. Photomicrograph, transmitted light.
FIGURE 8b. Microroentgenogram.
FIGURE 8c. Radioautograph. p.l. = pigmented layer; p.c.z. = principal calcified zone.

which passed through the pigmented layer and the principal calcified zone. In Figure 8a, the uniform gray area (p.c.z.) represents the principal calcified zone. The heavily pigmented, darker portion and the less heavily pigmented portion of the pigmented layer (p.l.) are visible.

In Figure 8b, the microroentgenogram of this section, the outermost portions of the pigmented layer appear to be heavily mineralized. The calcification suggesting interprismatic spaces is more clearly defined in the lower portion of the pigmented layer. In the principal calcified zone, the mineral is distributed in a uniform manner. The radioautograph, Figure 8c, shows again the irregular pattern of uptake of calcium-45 displayed by the carapace.

Discussion of photomicrographs, microroentgenograms, and radioautographs

Slight chemical differences have been observed among shell samples obtained from different areas of the exoskeleton of the lobster. The differences in density in these two areas apparent when Figures 4b and 5a are compared to Figure 6b could be a result of the slightly lower calcium concentration observed in the claw as compared to the carapace. The technique used to obtain the microroentgenograms is sufficiently sensitive to detect a difference of at least 15% in mineral density between two areas.

The irregular distribution of calcium-45 in the carapace, as compared to its distribution in the claw, suggests that the slightly denser mineral in the carapace contains a smaller percentage of labile calcium ions.

Drach (1939) pointed out that, in the integument of the crab, the area surrounding the hair pores may serve as a site for initial calcium deposits. The observation that some ducts and pores in the principal calcified zone are surrounded by denser mineral may bear some relation to Drach's finding.

The greater uptake of isotope by the pigmented layer, as compared to its uptake by the principal calcified zone, can be explained by the fact that the isotope was present in greater concentration at the outer surface of the shell.

General Summary and Conclusions

1. Visible light microscopy, microroentgenography, and radioautography were employed as techniques in the qualitative evaluation of the comparative distribution of mineral in the exoskeletons of lobsters and the relative exchangeability of calcium ions in the carapace and claw shells with the surrounding aqueous medium.

2. The following conclusions can be drawn from an examination of photographs obtained using these techniques: (1) In the claw and the carapace, a greater uptake of radiocalcium is observed in the outer portion of the shell. Radioactivity appears to be more uniformly distributed in the outer layer of the claw than in the same region in the carapace. (2) The outer third of the pigmented layer in the claw and the carapace contains the mineral material of greatest density. (3) In the claw, increased mineralization is observed in regions immediately surrounding the tegumental gland ducts and the hair pores; this increased mineralization is not observed in the carapace sections. The laminar nature of the principal calcified zone, observed in microroentgenograms of claw sections, is suggested only in carapace sections 50 microns in thickness. (4) The laminar nature of the pigmented layer of the carapace is obvious in microroentgenograms of cross-sections 50 microns in thickness.

LITERATURE CITED

BERGMAN, G., AND B. ENGFELDT, 1954. Studies on mineralized dental tissues. II. Microradiography as a method for studying dental tissue and its application to the study of caries. *Acta Odont. Scand.*, **12**: 99–132.

CLARK, E. P., AND J. B. COLLIP, 1925. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. *J. Biol. Chem.*, **63**: 461–464.

DENNELL, R., 1947. The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea Decapoda. *Proc. Roy. Soc. Lond., Ser. B*, **134**: 485–503.

DENNELL, R., 1960. Integument and exoskeleton. Pp. 449–472, *in:* The Physiology of Crustacea, Vol. 1, Academic Press, New York, New York.

DRACH, P., 1939. Mue et cycle d'intermue chez les crustacés decapodés. *Ann. Inst. Oceanog. Paris (N.S.)*, **19**: 103–391.

DUDICH, E., 1931. Systematische und biologische Untersuchungen über die Kalkeinlargerungen des Crustaceenpanzers in polarisiertem Lichte. *Zoologica, (Stuttgart)*, **80**: 1–154.

ENGSTRÖM, A., R. BJÖRNERSTEDT, C.-J. CLEMEDSON AND A. NELSON, 1957. Bone and Radiostrontium. John Wiley and Sons, New York, New York.

HERRICK, F. H., 1895. The American lobster, a study of its habits and development. *Bull. U. S. Fish Comm.*, **15**: 1–252.

PRENANT, M., 1927a. Recherches sur le calcaire chez les êtres vivants. La stabilité du calcaire amorphe et le tégument des Crustacés. *Ann. Physiol. Physico-Chim. Biol.*, **3**: 818–843.

PRENANT, M., 1927b. Les formes minéralogiques du calcaire chez les êtres vivants, et le problème de leur déterminisme. *Biol. Rev. Cambridge Phil. Soc.*, **2**: 365–393.

RICHARDS, A. G., 1951. The Integument of Arthropods. University of Minnesota Press, Minneapolis, Minnesota.

TRAVIS, D. F., 1955. The molting cycle of the spiny lobster, *Panulirus argus* Latreille. II. Pre-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. *Biol. Bull.*, **108**: 88–112.

TRAVIS, D. F., 1957. The molting cycle of the spiny lobster, *Panulirus argus* Latreille. IV. Post-ecdysial histological and histochemical changes in the hepatopancreas and integumental tissues. *Biol. Bull.*, **113**: 451–479.

TRAVIS, D. F., 1960. Matrix and mineral deposition in skeletal structures of the decapod crustacea. Pp. 57–116, *in:* Calcification in Biological Systems, AAAS Publication No. 64, Washington, D. C.

VITZOU, A.-N., 1882. Recherches sur la structure et la formation des téguments chez les crustacés decapodés. *Arch. Zool. Exp. Gén.*, **10**: 451–576.

WILLIAMSON, W. C., 1860. On some histological features in the shells of the crustacea. *Quart. J. Micr. Sci.*, **8**: 35–47.

YONGE, C. M., 1932. On the nature and permeability of chitin. 1. The chitin lining the foregut of decapod crustacea and the function of the tegumental glands. *Proc. Roy. Soc. Lond., Ser. B*, **111**: 298–329.

THORACIC NEUROSECRETORY STRUCTURES IN BRACHYURA. I. GROSS ANATOMY

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The pericardial plexus in decapod Crustacea was figured and described in several early investigations (Conant and Clark, 1896; Jolyet and Viallanes, 1893; Smith, 1947). Its significance, however, was not recognized until Alexandrowicz (1953) re-examined the structure, demonstrating that it contains not only nerve fibers, but also a number of arborizing terminations located just beneath the sheath surrounding the nerve trunks. This led to the suggestion, substantiated by later experiments (Alexandrowicz and Carlisle, 1953; Maynard and Welsh, 1959), that the pericardial plexus is the terminal portion of a neurosecretory system and is analogous to the sinus gland of the eyestalks. The plexus was renamed the pericardial organ (PO) on the basis of its secretory function.

Alexandrowicz's work left several aspects of the thoracic neurohaemal systems unresolved. This is the first of three papers which re-examine these systems in the brachyurans. It describes the anatomy of three neurohaemal structures in the crab thorax and suggests a correlation between PO volume and aspects of behavior. The second paper of the series will consider the histology and location of neurons contributing endings to the neurohaemal structures. The third paper will describe the histology of the terminations in the pericardial organs.

Methods

Nine species of crabs from five families were used: Cancridae, *Cancer borealis* (Stimpson); Xanthidae, *Panopeus herbstii* (Milne Edwards); Portunidae, *Callinectes ornatus* (Ordway); Grapsidae, *Grapsus grapsus* (L.), *Plagusia depressa* (Fabricious), *Goniopsis cruentata* (Latreille), *Pachygrapsus crassipes* (Randall), *Gecarcinus lateralis* (Fréminville); Ocypodidae, *Ocypode albicans* (Bosc). The *Cancer borealis* were obtained from Maine (Boothbay Harbor Crab and Lobster Co., Boothbay, Me.), the *Pachygrapsus crassipes* from California (Pacific Biomarine Supply Co., Los Angeles, Calif.), and both were examined during the winter at Ann Arbor. The other seven species were collected and examined at Bermuda during July and August.

Most of the following observations were made upon fresh, unstained material. Where necessary, certain points were confirmed with fresh material stained with methylene blue (Alexandrowicz, 1932) or with preserved specimens. In dissection the dorsal carapace was usually opened, and stomach, hepatopancreas, gonads, and bladder removed to expose the underlying ganglia and ventral thoracic structures. Complete and rapid removal of the hepatopancreas is critical—otherwise powerful digestive juices escape and destroy the finer nerve terminations—but is difficult

because of its delicacy and extensive ramifications. This problem was never completely solved, and prohibits definite statements about the absence of fine nerve branches or the ultimate destination of some nerves. Structures were considered neurosecretory if they appeared bluish-white with incident or darkfield illumination. Where there was doubt during dissection, the structure in question was removed and examined with high magnifications under darkfield illumination. Under these conditions, bluish or greenish colors can be considered a result of scattering or diffraction by small dense granules within the cytoplasm (see Maynard, 1961), and in the present series of investigations were found only in cell bodies, fibers, or terminals which could be termed neurosecretory on the basis of other criteria such as fine anatomy, staining characteristics, or physiological actions (see, also, Brown, 1940; Passano, 1953; Bliss and Welsh, 1952).

Pericardial organs were weighed individually on a fishpole balance constructed from a fine glass rod. One mm. deflection represented about 0.1 mg.; weights were estimated to the nearest 0.02 mg. In every case an attempt was made to include the entire PO but no non-PO material in the final weighing. This was generally believed successful.

Results

Three sites of storage and release of neurosecretory material are located in the crab thorax. Two, the pericardial organs and the anterior ramifications, apparently share some of the same secretory neurons, while the third, the post-commissure organ, is separate. The anatomy of the pericardial organs has been described by Alexandrowicz (1953) and others (Miyawaki, 1955; Matsumoto, 1958), but will be reviewed before proceeding to descriptions of the less known structures.

Pericardial organs

Structure. In the dozen or so brachyuran genera thus far examined, the pericardial organs show a common structural pattern. Typical organs from four species are diagrammed in Figure 1. In every case the PO appears as a plexus of nerve trunks in the lateral pericardium. Following the terminology of Alexandrowicz, this plexus may be divided into an anterior bar region and a posterior bar region, the two being joined by longitudinal trunks. Three nerve trunks pass into the anterior bar region from the ventral nerve mass. The two most anterior enter the lumen of the first branchio-pericardial (b-p) vein some distance from the pericardium; the third enters the lumen of the second b-p vein near its opening to the pericardium. These trunks, with occasional side twigs, run freely in the blood channels to the pericardium where they unite either completely to form a true anterior bar (in *Maia* and *Libinia*) or only partially to form a network of bars (most other genera, Figure 1) which lies across the combined opening of the first and second branchio-pericardial veins. Two nerves pass dorsally from the anterior bar region, one going to the dorsal thoracic muscles and one to the heart as the *dorsal nerve* (Alexandrowicz, 1932). There may be two (*Ocypode, Carcinus, Libinia*) or three longitudinal trunks joining the anterior and posterior bars. These trunks, like the bar regions, may float freely in the pericardial sinus, but often their posterior third is bound to the pericardial wall. The posterior bar lying over the opening of the third branchio-pericardial vein may be simple or complex in form.

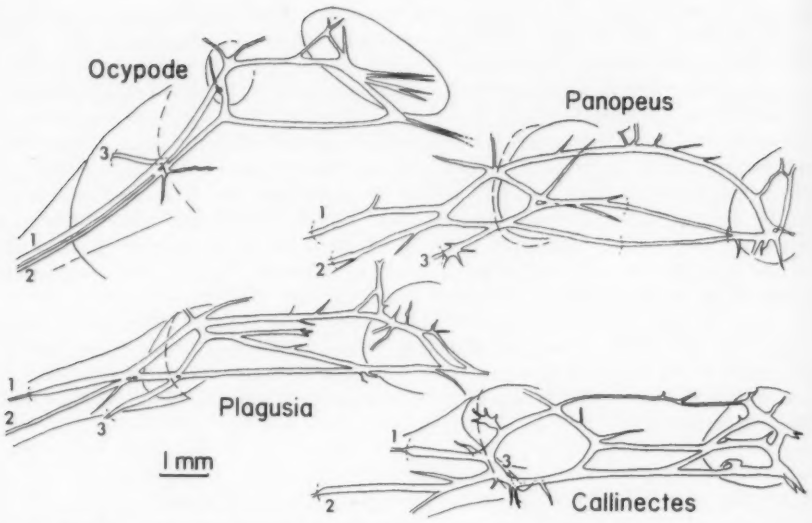

FIGURE 1. Pericardial organs of *Ocypode albicans, Panopeus herbsti, Plagusia depressa,* and *Callinectes ornatus.* Drawings from fresh preparations, organs *in situ* with openings of branchio-pericardial veins indicated. Anterior is to the left. Short dashed lines crossing nerves indicate point of egress from the pericardium or vein, or, in *Panopeus,* regions of two lower trunks which fuse with pericardial wall. The numbers 1, 2, and 3 indicate the first, second, and third segmental nerves, respectively. In the anterior bar region of *Plagusia,* three ovals mark position of anterior neuron cell bodies.

A number of trunks lead from it, some apparently going to the dorsal musculature and some to the ventral thoracic nerve mass as segmental nerves. Little attention has been given these in the present work, but at least some of the branches have been traced and described in *Pugettia* (Heath, 1941). As indicated below, the surface of all free-floating trunks may be considered a potential secretory surface. In addition, some portions of the pericardial lining and ligaments may contain secretory nerve terminations (Alexandrowicz, 1953). These latter areas, however, seem of less importance in the Brachyura than in the Astacura.

Size. Preliminary measurements on *Cancer, Carcinus,* and *Libinia* (Maynard and Welsh, 1959) suggested a correlation between relative pericardial organ weight and habitat or behavior of the species. Weights of pericardial organs were accordingly determined in a series of seven semi-tropical species from varying habitats. The results are given in Table I. The corrected pericardial organ index (weight of one PO divided by body weight, where body weight represents total weight *minus* chelipeds) is significantly higher in active swimming or semi-terrestrial forms such as *Callinectes* and *Ocypode* than in the more sluggish mud crab, *Panopeus.* Intertidal forms such as *Grapsus* and *Plagusia,* or less active terrestrial forms such as *Gecarcinus* fall between (Table II). The PO index showed no systematic variation with sex or with body weight within the samples examined. It may be of interest, however, that the uncorrected PO index (PO weight divided by total

TABLE I

Weight of pericardial organs (PO) in semi-tropical Brachyura

Animal	Habitat	n	Body wt. (gm.)	Total wt. (gm.)	PO wt. (mg.)	PO/BW (10^{-5})	PO/TW (10^{-5})
Ocypode	sandy beaches, above high tide	10	8.1–26.1	14.2–49.4	0.12–0.36	1.58±.23	0.90±.14
Callinectes	shallow bays, brackish	5	36.0–63.4	54.3–104.5	0.50–1.16	1.54±.35	0.99±.23
Gercarcinus	land, burrows	13	16.6–27.1	27.9–48.2	0.12–0.42	1.44±.42	0.87±.24
Goniopsis	mangrove swamps, intertidal	7	8.9–28.7	14.8–55.8	0.12–0.38	1.34±.11	0.76±.11
Plagusia	rocky shores, just below water	10	32.9–50.4	49.8–89.2	0.42–0.72	1.29±.11	0.80±.11
Grapsus	rocky shores, just above water	9	25.6–52.6	42.3–102.7	0.32–0.62	1.23±.17	0.68±.11
Panopeus	shallow bays, muddy bottoms	10	13.8–34.9	26.4–69.9	0.08–0.26	0.70±.21	0.39±.12

TABLE II

Significance levels of comparisons between PO indices of seven Brachyura; determined for corrected (upper) and uncorrected (lower) PO indices by Student's t test

Corrected index: PO/BW

	Ocypode	*Callinectes*	*Gecarcinus*	*Goniopsis*	*Plagusia*	*Grapsus*	*Panopeus*
Ocypode	—	ns	ns	$p = .025$	$p = .005$	$p = .005$	$p < .0005$
Callinectes	ns	—	ns	ns	$p = .05$	$p = .05$	$p < .0005$
Gecarcinus	ns	ns	—	ns	ns	ns	$p < .0005$
Goniopsis	$p = .05$	$p = .05$	ns	—	ns	ns	$p < .0005$
Plagusia	ns	$p = .05$	ns	ns	—	ns	$p < .0005$
Grapsus	$p = .005$	$p = .005$	$p = .025$	ns	$p = .025$	—	$p < .0005$
Panopeus	$p < .0005$	$p < .0005$	$p < .0005$	$p < .0005$	$p < .0005$	$p < .0005$	—

Uncorrected index: PO/TW

TABLE III

Weight of pericardial organs in temperate Brachyura (Maynard and Welsh, 1959)

Species	Habitat	Mean body wt. (gm.)	Mean PO wt. (mg.)	PO/BW (10^{-5})
Carcinus maenas	Maine coast, intertidal	102	0.54	0.53
Cancer borealis	Maine coast, subtidal	351	1.00	0.30
Cancer irroratus	Maine coast, subtidal	214	0.54	0.28
Cancer pagurus	North sea, subtidal	1810	4.0	0.22
Libinia emarginata	Mass. coast, subtidal	647	1.6	0.25
Maia squinado	North sea, subtidal	1560	2.5	0.16

weight, including claws) in the temperate or northern species examined was generally less than that of analogous semi-tropical forms (Table III).

Several factors require that interpretation of the above correlations be made with caution. First, the significant behavioral variable is not certain. Second, histological studies to be reported elsewhere (Maynard and Maynard, unpublished data) indicate that the size of the organ does not necessarily reflect the amount of secretory material contained within it. And third, the pericardial organs do not necessarily represent the only locus for storage and release of neurosecretion in the thorax, and their size alone, therefore, does not reflect the total presumptive storage volume. It seems probable that further information on the physiological function of secretions released from the PO will be necessary before a reasonable evaluation of the significance of the PO index can be made.

Anterior ramifications

Structure. The three antero-ventral trunks of the pericardial organs originate as segmental nerves in the ventral nerve mass. The most anterior of these, the first segmental nerve, corresponds to the cardio-inhibitory nerve, and the second and third to the first and second cardio-acceleratory nerves (Table IV). They leave the ventral ganglia at the level of the second, third, and fourth thoracic segments. The accelerator nerves apparently pass to the pericardium without extensive branching; they contribute fibers to the PO and one fiber each to the dorsal nerve innervating the cardiac ganglion. The inhibitor nerve, however, branches extensively in the region of the muscles supplying the maxillae. This branching forms the *anterior ramifications* (AR), and represents a second peripheral secretory structure (Figs. 2, 3 and 4). A detailed description follows.

Upon emerging from the ventral ganglion mass just behind the integumentary nerve, the first segmental nerve (inhibitor nerve) and integumentary nerve travel

TABLE IV

Terminology of anterior dorsolateral nerves in the decapod thorax

Genus	Author	Terminology			
Astacus	Keim, 1915	nervus superior primus	nervus superior secundus	nervus superior tertius	nervus superior quartus
Cambarus	Chaudonneret, 1956	nervus superior primus	nervus superior secundus	nervus superior tertius	nervus superior quartus
Cambarus	Wiersma and Novitski, 1942	—	inhibitor nerve	accelerator n.	—
Procambarus	Wiersma and Pilgrim, 1961	—	2nd root, 1st thoracic segment (t.s.)	2nd root, 2nd t.s.	2nd root, 3rd t.s.
Panulirus	Maynard, 1953	—	cardio-inhibitor nerve	first cardio-accelerator n.	second cardio-accelerator n.
Pugettia	Heath, 1941	integumentary nerve	segmental cardiac nerve x	segmental cardiac nerve m	segmental cardiac nerve I
Cancer	Smith, 1947	recurrent cutaneous nerve	inhibitory cardiac nerve	excitatory cardiac nerve	excitatory cardiac nerve
Brachyura	this paper	integumentary nerve	segmental nerve, one	segmental nerve, two	segmental nerve, three

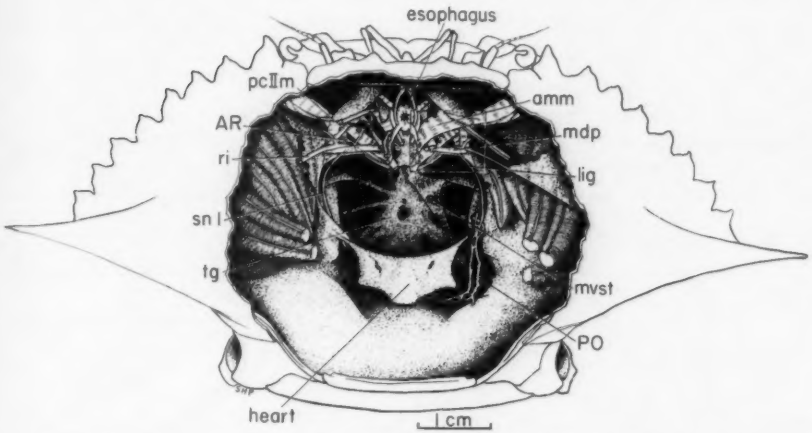

FIGURE 2. Dissection of preserved *Callinectes ornatus* showing location of pericardial organs and anterior ramifications. Digestive gland, stomach, and bladder removed. Amm, musculus abductor maior mandibulae; AR, anterior ramifications; lig, ligament; mdp, musculus dorsoventralis posterior; mvst, musculi ventrales superficiales thoracis; pcIIm, musculus proximalis coxopoditis II maxillae; PO, pericardial organ; ri, recurrent integumentary nerve; sn 1, first segmental nerve; tg, thoracic ganglia. The region bounded by a rectangle to the right of the esophagus is enlarged in Figure 3. Several overlying muscles and ligaments have been removed on the left half of the dissection.

anteriorly and laterally together to the endopleurite of the last head segment. There they pass upward through a ligament loop and angle more laterally toward the musculus dorsoventralis posterior (mdp). The integumentary nerve is often medial and anterior to the segmental nerve at this point. After passing through the ligament loop, and usually before passing beyond the mdp, the first segmental nerve gives off two branches. One angles posteriorly to the muscles attached to ligaments overlying the ventral nerve mass (musculi ventrales superficiales thoracis, Fig. 6), the other larger branch runs anteriorly beneath and beyond the mdp where it proceeds to break up into a network or plexus, the anterior ramifications. These apparently lie beneath a sinus membrane separating the ventral respiratory muscles from the dorsal thoracic cavity. Portions of the AR may dip ventrally in blood spaces between the muscles, or may spread over the membranes covering skeletal elements in the region or over the surface of a nerve (Fig. 3) apparently passing out toward the Y-organ and muscles of the mandible (Echalier, 1959). The form of the AR varies among species; for example, in *Callinectes* the ramifications give the impression of two main branches (Figs. 2 and 3), while in *Plagusia* (Fig. 4) they resemble an irregular net with no obvious main trunk. Most of the branchings and twigs of the ramifications appear to lead nowhere, as in the PO, but there are at least two branches, A and B, in which this is not so. These are particularly obvious in *Callinectes, Plagusia,* and *Eupanopeus*. Branch A separates under the mdp and, travelling medially, appears to enter this muscle near its medial attachment. Its final terminations have not been found. Branch B passes anterolaterally from the ramifications, paralleling the anterior branch of the integumentary nerve.

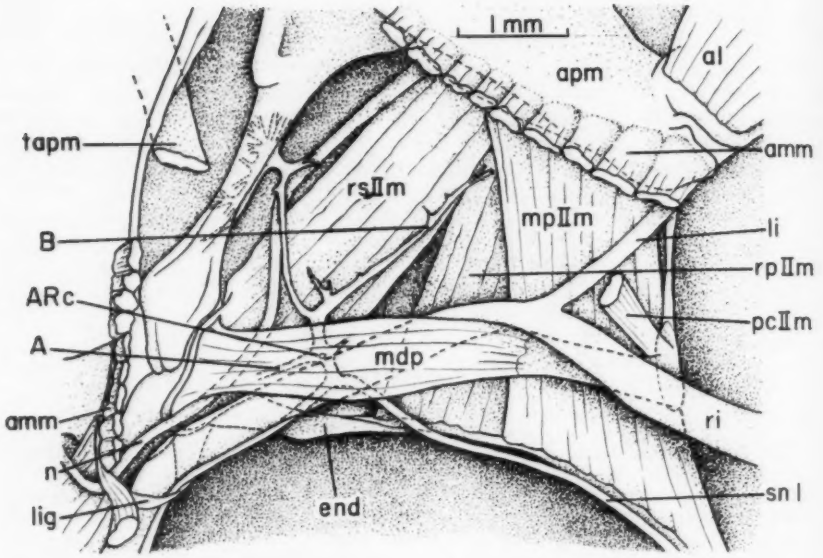

FIGURE 3. Anterior ramifications of *Callinectes ornatus;* fresh material. Dorsal exposure with digestive system removed. A, medial, posterior branch of anterior ramifications; al, musculus adductor lateralis mandibulae; amm, musculus abductor maior mandibulae, only cut segments at origin and insertion remain; the belly has been removed to expose underlying structures. Apm, apophysis of mandible; ARc, location of neuron cell body in anterior ramifications; B, anterior, lateral branch of anterior ramifications; end, endopleurite of last head segment; li, lateral integumentary nerve; lig, ligament; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; n, anterior dorsolateral nerve of Echalier (1959); pcIIm, musculus proximalis coxopoditis II maxillae; ri, recurrent integumentary nerve; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn I, first segmental nerve; tapm, stump of tendon of musculus adductor posterior mandibulae. Note how one anterior branch of anterior ramifications spreads over anterior nerve and continues beneath the head apodeme.

In *Ocypode* this branch leads to another more lateral ramification (Fig. 5), but in other species the second ramification seems lacking and branch B disappears among muscles and membranes of the lateral, anterior carapace.

The AR are obviously located in a portion of the venous sinus system. Although the course of haemolymph flow has not been accurately determined in these regions, fluid probably passes over the ramifications as it travels from the general anterior venous sinuses of the thorax to the branchial sinuses in the gills. Perhaps of equal significance is the location of the AR in close proximity to the major respiratory muscles of the scaphognathite. Between them, therefore, the AR and PO are located just upstream from those muscles—respiratory muscles and heart, respectively—which normally exhibit continuous, rhythmic contractions and which are most concerned in the maintenance of adequate oxygen supply to the tissues of the body.

The volume of the AR is less than that of the PO, but its relative size was not measured because of difficulties in removing it without fragmentation.

Post-commissure organs

Although post-commissure organs have been identified in various shrimp and Stomatopoda, there is no published record of their presence in Brachyura. The following account describes them in *Pachygrapsus crassipes* (Fig. 6).

In *Pachygrapsus,* as in most other decapods, a pair of post-commissural nerves

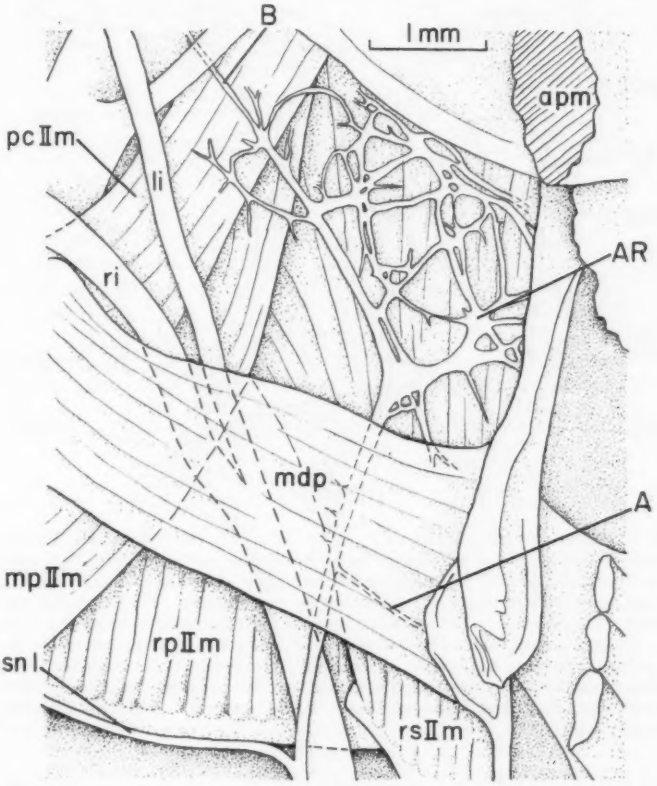

FIGURE 4. Anterior ramifications of *Plagusia depressa;* fresh material. Dorsal exposure with digestive system removed; midline to right. A, medial, posterior branch of anterior ramifications; apm, stump of apophysis of mandible; AR, anterior ramifications; B, anterior, lateral branch of anterior ramifications; li, lateral integumentary nerve; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; pcIIm, musculus proximalis coxopoditis II maxillae; ri, recurrent integumentary nerve; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve.

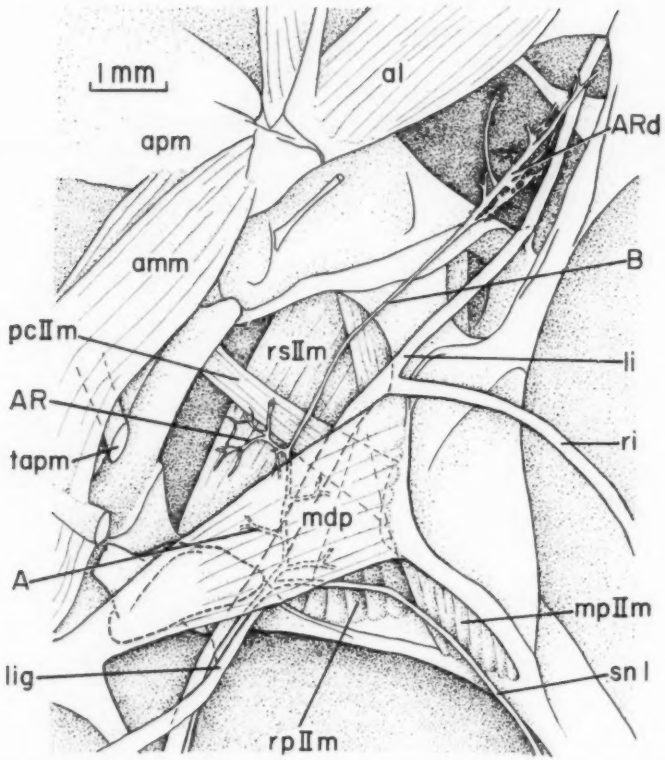

FIGURE 5. Anterior ramifications of *Ocypode albicans;* fresh material. Dorsal exposure with digestive system removed, midline to left. A, medial, posterior branch of anterior ramifications; al, musculus adductor lateralis mandibulae; amm, musculus abductor maior mandibulae (intact); apm, apophysis of mandible; AR, anterior ramifications; ARd, anterior ramifications, distal segment; B, anterior, lateral branch of anterior ramifications; li, lateral integumentary nerve; lig, ligament; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; pcIIm, musculus proximalis coxopoditis II maxillae; ri, recurrent integumentary nerve; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve; tapm, stump of tendon of musculus adductor posterior mandibulae.

arises from the post-esophageal commissure. Each of these passes dorsally through the posterior esophageal muscles where it is joined by a major branch of a nerve originating in the homolateral circumesophageal ganglion (see also Fig. 5; Heath, 1941). There are a number of smaller branches from these two nerves which innervate the surrounding esophageal muscles (Fig. 6). In view of the observation that in shrimp the post-commissural nerve does not send fibers to these muscles (Knowles, 1953), it seems possible that a similar situation exists in the Brachyura, and that nerve fibers innervating the esophageal muscles are derived from the nerve

originating in the circumesophageal ganglia and not the post-commissural nerve. The latter nerve passes from the post-esophageal muscles with no apparent diminution in size, and, after travelling laterally for a short distance, divides into two equal branches. One of these goes forward to terminate in a network of fibers spreading over the anterior third of the ligamentum ventrale capitis. This network is homologous with the lamellae of the post-commissure organ complex in shrimp and presumably constitutes the storage-release structure analogous to the AR and PO. It

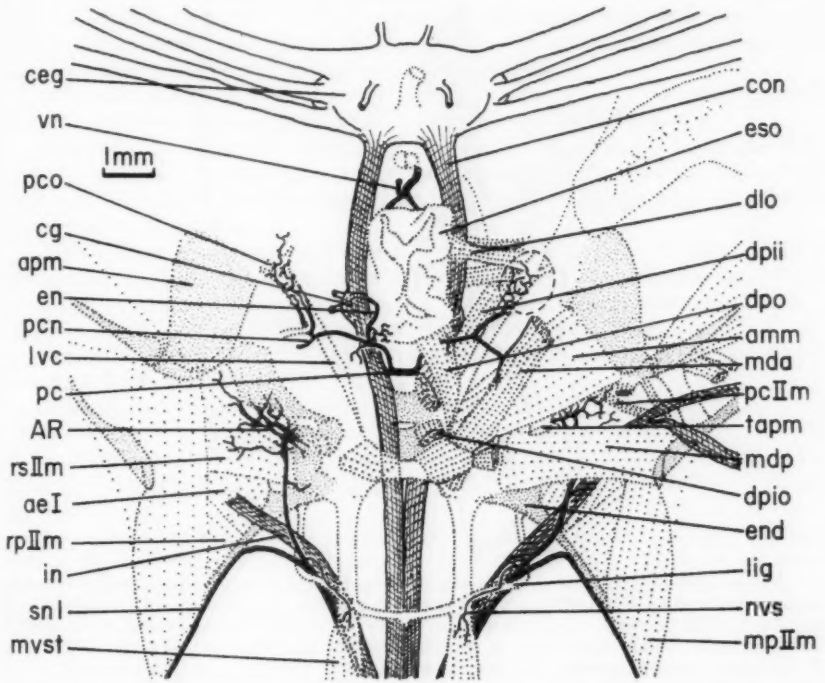

FIGURE 6. Anterior ramifications and post-commissure organs of *Pachygrapsus crassipes;* semi-diagrammatic, from fresh preparation. Dorsal exposure, digestive system removed. Ae I, musculus attractor epipoditis I pedis maxillaris; amm, musculus abductor maior mandibulae, apm, apophysis of mandible; AR, anterior ramifications; ceg, cerebral ganglia; cg, connective ganglion; con, circumesophageal connective; dlo, musculus dilatator lateralis oesophagei; dpii, musculus dilatator internus pylorici inferior; dpio, musculus dilatator externus pylorici inferior; dpo, musculus dilatator posterior oesophagei; en, nerve to esophageal muscles; end, endopleurite of last head segment; eso, esophagus; in, integumentary nerve; lig, ligament; lvc, ligamentum ventrale capitis; mda, musculus dorsoventralis anterior; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; mvst, musculi ventrales superficiales thoracis; nvs, nerve to ventral superficial muscles; pc, post-esophageal commissure; pcIIm, musculus proximalis coxopoditis II maxillae; pcn, post-commissure nerve; pco, post-commissure organ; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve; tapm, stump of tendon of musculus adductor posterior mandibulae; vn, nerves of visceral system. The oval bounded by dashed lines above the right post-commissure organ represents the location of a dorsoventral venous channel.

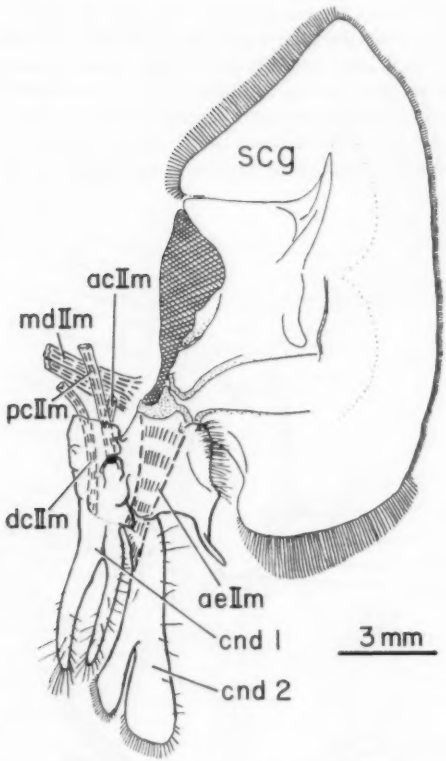

FIGURE 7. Second maxilla of *Callinectes*, showing muscles of coxopodite. AcIIm, musculus adductor coxopoditis II maxillae (no. 60, Cochran); aeIIm, musculus adductor endopoditis II maxillae (no. 61, Cochran); cnd 1, first endite of coxopodite; cnd 2, second endite of coxopodite; dcIIm, musculus distalis coxopoditis II maxillae; mdIIm, musculus depressor II maxillae (no. 59, Cochran); pcIIm, musculus proximalis coxopoditis II maxillae; scg, scaphognathite.

contains secretory granules and will be considered the post-commissure organ. The second branch of the post-commissural nerve innervates a muscle tentatively identified as the musculus dorsoventralis anterior (mda). This is possibly homologous with the "moulting muscle" in shrimp (Knowles, 1953).

In general, the entire anterior-ventral portion of the crab thorax is filled with stomach, hepatopancreas, or ramifications of the bladder. Venous haemolymph flow must therefore be largely confined to channels or spaces among the various viscera. The anterior third of the post-commissure organ is located across such a channel, outlined with dashes in Figure 6, which passes dorsoventrally between bladder protuberances beneath the stomach. Like the other neurohaemal structures of the thorax, the post-commissure organ is in a position to be continually bathed by flowing, venous haemolymph.

In *Pachygrapsus* the post-commissure organ appears the smallest of the three known thoracic neurohaemal structures.

Thoracic musculature

In the course of dissection, several muscles and ligaments were found in the anterior thorax which have not been generally recognized in the Brachyura. Since some of these are in intimate contact with the neurohaemal structures and have been used in the above descriptions or shown in figures, they are listed below with brief mention of their location. Muscles presumed homologous with those described in Macrura are named accordingly (Balss, 1941). Muscles whose homologies are uncertain are named according to their site of insertion.

Musculus dorsoventralis posterior (mdp) (Figs. 2, 6). Origin: ligaments above cephalic apodeme, runs laterally. Insertion: lateral carapace.

Musculus dorsoventralis anterior (mda) (Fig. 6). Origin: ligaments above cephalic apodeme, more medial and posterior than mdp, runs dorso-anteriorly beside stomach. Insertion: dorsal carapace just behind sockets of eyestalks (observed in *Pachygrapsus* and *Cancer*).

Ligamentum (*musculus*) *ventrale capitis* (lvc) (Fig. 6). Origin: ligaments above cephalic apodeme, runs anteriorly and slightly laterally. Insertion: carapace at origin of lateral muscles to esophagus. No muscle fibers were found in this structure in the two species examined, *Pachygrapsus* and *Cancer*.

Musculi ventrales superficiales thoracis (mvst) (Figs. 2, 6). Thin, flat muscles overlying thoracic nerve mass in thorax.

Musculus proximalis coxopoditis II maxillae (pcIIm) (Figs. 2, 3, 5, 6, 7). Origin: lateral carapace just anterior to insertion of mdp, runs anteriorly, medially, and ventrally. Insertion: inner membrane of coxopodite of second maxilla (Fig. 7). Apparently pulls coxopodite anteriorly and turns toward mouth (not figured by Cochran, 1935).

Musculus distalis coxopoditis II maxillae (dcIIm) (Fig. 7). Origin: beneath muscle 59, *musculus depressor II maxillae* (Cochran, 1935). Insertion: on medial side of coxopodite of second maxilla, distal to pcIIm. Apparently pulls coxopodite posteriorly and toward mouth (not figured by Cochran, 1935).

Discussion

The observations reported show that the peripheral neurosecretory complex in the brachyuran thorax is much more elaborate than heretofore supposed. Not only are post-commissure organs present as in the Macrura (Knowles, 1953), but the major thoracic neurohaemal structures, the pericardial organs, are but part of a still more extensive system which includes the anterior ramifications.

The total presumptive storage volume for secretion is also relatively large, as may be seen from a comparison between pericardial organs and sinus gland. The PO are the largest neurohaemal structures in the thorax, ranging from 0.25 to 1.0 mg. per 100 gm. total body weight. In *Carcinus maenas* the secretory terminations in the PO trunks form an outer layer which is about 10% of the trunk radius in thickness (Maynard and Maynard, unpublished data). The actual presumptive storage volume of the PO is therefore about one-fifth its total volume, 0.05 to 0.20

mg./100 gm. This compares favorably with the volume of the sinus gland of the fresh-water crayfish, *Cambarus virilis*, 0.2 to 0.45 mg./100 gm. total body weight (Brown, 1940).

Since functions of the thoracic structures, the PO-AR system in particular, are currently uncertain, some speculation from anatomy seems justified. Originally, the location of the PO in the pericardium led to the suggestion of a role in cardio-regulation (Alexandrowicz, 1953). Experiments showing cardio-acceleration by PO extracts supported the suggestion (Alexandrowicz and Carlisle, 1953; Maynard and Welsh, 1959). It was recognized, however, that the position of the PO is also perfectly suited for release of materials into the haemolymph for immediate distribution over the entire body, and the possibility of more general functions for PO secretion was postulated (Maynard and Welsh, 1959). The present work strengthens the latter possibility. Not only is the thoracic system remarkably extensive and voluminous for a cardio-regulatory structure, but branches of the first segmental nerve enter both the PO and AR and single neurons probably have secretory terminations in both structures (Maynard, 1961). This does not seem efficient if such neurons are concerned only with cardio-regulation, for the AR is located in a passage to the anterior branchial sinus and is separated from the heart by the full length of the afferent and efferent branchial veins. Such an arrangement is reasonable, however, if PO-AR secretion were indirectly concerned with respiratory transport. In addition to increased heart rate, one might presume that secretions from the AR portion of the complex play some role in oxygen uptake or circulation in the gills. Two possibilities may be mentioned. First, AR secretion may affect ventilation in the gill chamber by acting on scaphognathite muscle activity. A common hormone affecting both the heart and scaphognathite muscles could provide the loose coordination desirable for optimum gaseous exchange between tissue and external medium. Second, and perhaps more attractive, AR secretion may alter resistance to haemolymph flow in gill veins (Parrot, 1938). PO-AR secretions would therefore increase turn-over time by simultaneously increasing heart rate and reducing peripheral resistance. Increased turnover time would in turn tend to increase oxygen transport rates.

As mentioned earlier, the correlations found between PO volume and apparent activity of the brachyurans examined are interesting, but are very difficult to interpret. They are in line with the thesis that PO-AR secretions have some function in metabolism, water balance, or stress reactions, but hardly can be accepted as positive evidence. Appropriate comparative studies on the physiology of the brachyurans involved have not been published.

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Summary

1. Three neurohaemal structures occur in the crab thorax: the pericardial organs (PO) in the pericardium; the anterior ramifications (AR) in a ventral sinus near

the respiratory muscles; and the post-commissure organs, lateral to the esophagus and ventral to the stomach. Since the first segmental nerve supplies both PO and AR, the two are considered separate release sites of a single secretory complex.

2. The PO ranges between 0.00025 and 0.001% of total body weight in Bermuda crabs. It is largest in the most active species.

LITERATURE CITED

ALEXANDROWICZ, J. S., 1932. The innervation of the heart of the Crustacea. I. Decapoda. *Quart. J. Micr. Sci.*, **75**: 181–249.

ALEXANDROWICZ, J. S., 1953. Nervous organs in the pericardial cavity of the decapod Crustacea. *J. Marine Biol. Assoc.*, **31**: 563–580.

ALEXANDROWICZ, J. S., AND D. B. CARLISLE, 1953. Some experiments on the function of the pericardial organs in Crustacea. *J. Marine Biol. Assoc.*, **32**: 175–192.

BALSS, H., 1941. Decapoda. *In:* "Bronn's Tierreich," Bd. 5, Apt. 1, Bch. 7, Lfg. 2. Akademische Verlags., Leipzig.

BLISS, D. E., AND J. H. WELSH, 1952. The neurosecretory system of brachyuran Crustacea. *Biol. Bull.*, **103**: 157–169.

BROWN, F. A., JR., 1940. The crustacean sinus gland and chromatophore activation. *Physiol. Zoöl.*, **13**: 343–355.

CHAUDONNERET, J., 1956. Le système nerveux de la région gnathale de l'écrevisse, *Cambarus affinis* (Say). *Annal. Sci. Nat. Zool.*, **18**: 33–61.

COCHRAN, D. M., 1935. The skeletal musculature of the blue crab, *Callinectes sapidus* Rathbun. *Smithson. Misc. Coll.*, **92**: no. 9.

CONANT, F. S., AND H. L. CLARK, 1896. On the accelerator and inhibitor nerves to the crab's heart. *J. Exp. Med.*, **1**: 341–347.

ECHALIER, G., 1959. L'organe Y et le déterminisme de la croissance et de la mue chez *Carcinus maenas* (L.), crustacé décapode. *Ann. Sci. Nat. Zool., 12th ser.*, **1**: 1–57.

HEATH, J. P., 1941. The nervous system of the kelp crab, *Pugettia producta*. *J. Morph.*, **69**: 481–498.

JOLYET, F., AND H. VIALLANES, 1893. Recherches physiologiques sur le système nerveux accélérateur et moderateur du coeur chez le crabe. *Ann. Sci. Nat. Zool.*, **14**: 387–404.

KEIM, W., 1915. Das Nervensystem von *Astacus fluviatilis* (*Potamobius astacus* L.). Ein Beitrag zur Morphologie der Dekapoden. *Zuitschr. wiss. Zool.*, **113**: 485–545.

KNOWLES, F. G. W., 1953. Endocrine activity in the crustacean nervous system. *Proc. Roy. Soc. London, Ser. B*, **141**: 248–267.

MATSUMOTO, K., 1958. Morphological studies on the neurosecretion in crabs. *Biol. J. Okayama Univ.*, **4**: 103–176.

MAYNARD, D. M., 1953. Activity in a crustacean ganglion. I. Cardio-inhibition and acceleration in *Panulirus argus*. *Biol. Bull.*, **104**: 156–170.

MAYNARD, D. M., 1961. Thoracic neurosecretory structures in Brachyura. II. Secretory neurons. *Gen. Comp. Endocrin.* (in press).

MAYNARD, D. M., AND J. H. WELSH, 1959. Neurohormones of the pericardial organs of brachyuran Crustacea. *J. Physiol.*, **149**: 215–227.

MIYAWAKI, M., 1955. Observations on the pericardial organ in two kinds of crabs, *Paralithodes brevipes* Brandt and *Telmessus cheiragonus* Tilesius. *Dobutsugaku Zasshi*, **64**: 137–140.

PARROT, J. L., 1938. Propriétés des extraits alcooliques de système nerveux et de certains viscères de *Maia squinado*. *C. R. Soc. Biol.*, **129**: 277–279.

PASSANO, L. M., 1953. Neurosecretory control of molting in crabs by the X-organ sinus gland complex. *Physiol. Comp. et Oecol.*, **3**: 155–189.

SMITH, R. I., 1947. The action of electrical stimulation and of certain drugs on cardiac nerves of the crab, *Cancer irroratus*. *Biol. Bull.*, **93**: 72–88.

WIERSMA, C. A. G., AND E. NOVITSKI, 1942. The mechanism of the nervous regulation of the crayfish heart. *J. Exp. Biol.*, **19**: 255–265.

WIERSMA, C. A. G., AND R. L. C. PILGRIM, 1961. Thoracic stretch receptors in crayfish and rock lobster. *Comp. Biochem. Physiol.*, **2**: 51–64.

GLUTATHIONE-CONTROLLED ANAEROBIOSIS IN CRYPTOCERCUS, AND ITS DETECTION BY POLAROGRAPHY[1, 2]

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The flagellate protozoa of *Cryptocercus punctulatus*, hind-gut symbionts of this wood-feeding roach, are generally regarded as obligate anaerobes. Cleveland *et al.* (1934) and Cleveland and Burke (1956) reported high oxygen susceptibility for *Cryptocercus* protozoa during *in vivo* studies relating oxygen toxicity with tension and temperature changes. Closely related termite protozoa are also killed by oxygen, demonstrated *in vivo* (Cleveland, 1925a, 1925b; Yamaski, 1931) and *in vitro* (Trager, 1933; Hungate, 1939). Trager's culture experiments with termite (*Zootermopsis*) protozoa, however, indicated to him that one flagellate species, *Trichonympha sphaerica*, probably requires oxygen in a very low but definite concentration.

It has been proposed that anaerobic status, *in strictu sensu*, might be assigned hind-gut-residing termite and roach protozoa if protozoa survival accompanied host survival following prolonged exposure of the host to nitrogen. Gilmour (1940) observed flagellate death in termites exposed to nitrogen and concluded that these protozoa are killed in their own metabolic end-products rather than from oxygen lack. Unpublished studies (Ritter) using *Cryptocercus* support Gilmour's conclusion. In brief, rapid cell destruction follows organic acid accumulation and pCO_2 rise with subsequent hind-gut fluid loss.

In all of these experiments it is impossible to define the exact status of oxygen (whether applied in excess of atmospheric concentration or totally omitted) and its influence upon the protozoa. Complex and critical side effects induced by secondarily altered chemical and physical constants must be assumed. Furthermore, it has not been established that a minute trace of oxygen might not be present, adequate, and quite essential for protozoan requirements. Indeed it is difficult to visualize a hind-gut content totally anaerobic considering its confinement in tissues liberally supplied with tracheal endings.

The immediate need, before further attempts to isolate the exact oxygen effect upon the protozoa of *Cryptocercus*, was development of a medium for *in vitro* culture. Actual design of the *in vitro* culture, however, was predicated upon a more certain understanding of the oxygen concentration, if any, present in roach hind-gut content.

The following study involves an apparently successful attempt to qualify anaerobiosis with respect to degree in very small samples of *Cryptocercus* hind-gut

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content with the highly oxygen-sensitive polarograph. Related phenomena are described and discussed to the extent present evidence will allow.

Materials and Methods

1. *The experimental animal*

A large collection of *Cryptocercus* was maintained at 20° C. in a metal container. Individuals dwelled within and fed upon portions of the same partially decomposed wood in which they were found. Only adult roaches were selected to supply material for the polarographic study. Hind-gut fluid was removed from the roach by holding it between thumb and forefinger, and squeezing with pressure strokes in an antero-caudad direction on the abdominal region. For a description of this insect as well as the protozoan symbionts it contains, see L. R. Cleveland *et al.*, (1934).

2. *Polarography*

Polarography, utilizing an apparatus with a sensitivity limit of approximately 10^{-6} M (0.001 mM) is one of the most efficient and accurate methods for oxygen detection (Kolthoff and Lingane, 1952). The normal hind-gut volume of an adult roach approximating only 0.1 ml., an abundance of suspended cellular and cellulose constituents in a brown and rather viscous liquid, and the likelihood of encountering a very low partial pressure of oxygen are factors which initially eliminated other techniques from consideration.

A Sargent Model XI recording polarograph was used in conjunction with a micro H-cell, possessing a solution chamber functional with slightly less than 0.1 ml. of sample. Although the micro-cell was designed and constructed with a side arm loading port suggested by Beecher, Follansbee, Murphy and Craig (1942), its use as a means of protecting the sample from air during loading proved to be unnecessary in this work. A constant flow of nitrogen was maintained while the roach hind-gut substance was squeezed into the micro-cell, during insertion of the mercury-dropping electrode, and for duration of the analysis.

Preparation of the saturated KCl-agar gel used in the salt bridge of the micro-cell and the saturated KCl solution for use in the reference electrode was performed according to Kolthoff and Lingane's method (1952). This reference and that of L. Meites (1955) offer detailed reviews of polarographic theory.

A polarogram is a plot of the current flowing through the solution in a polarographic cell against the potential of the mercury-dropping electrode. Polarograms used to document this study were traced and arranged to follow generally the order of discussion.

All polarograms were made with the same dropping electrode capillary at a constant rate of mercury drop formation. The 20° C. water bath surrounding the electrolysis vessel and all other operating conditions involved in polarographic technique were held constant.

Since the comparative method of wave height-concentration plot was used, rather than the Ilkovič equation to calculate the concentration of substance present, it is necessary to note that not all polarograms included could be recorded at the same sensitivity.

In order to record a high substance concentration, a polarograph setting was selected (*e.g.* shunt X-5 or X-10), resulting in lower apparatus sensitivity. The full wave height formed between the residual current (the lower plateau of the polarogram) and the limiting current (the upper plateau of the polarogram) wave portions, therefore, could be included within the polarographic paper margins. A low substance concentration could be recorded conveniently at a polarographic setting of shunt X-1 or X-2 while utilizing maximum apparatus sensitivity. The shunt setting, used in recording a polarogram, is included in legend data corresponding to the lettered curve figured. When a quantitative comparison is made between two polarograms, concentration reading corrections must be made for polarograms recorded at different sensitivity settings. For example, in a polarogram recorded at an apparatus shunt setting X-2, sensitivity would be one-half that utilized in a record made at a setting of X-1. Likewise, a setting X-1 indicates a recording sensitivity five times greater than a setting X-5. The wave height (corrected when necessary as directed) represents concentration, and this is read in microampere units of current. The voltage range in all polarograms extends from 0 to −0.4 volt.

3. *Glutathione assay*

The procedure used for a sodium nitroprusside assay of reduced glutathione was developed by Grunert and Phillips (1951), although in this study sodium chloride and metaphosphoric acid were not included as in Benesch, Benesch and Rogers (1953).

The alloxan (alloxan monohydrate, Eastman) "305" method of colorimetric determination, according to Patterson, Lazarow and Levey (1949), was modified only to the extent of reducing reaction volumes for adaptation to the Beckman-Spinco ultramicro analytical system. Application of the Beckman DU spectrophotometer to minute quantity measurement is described by Lowry and Bessey (1946).

The microbioassay for reduced glutathione, based on the "feeding reaction" of *Hydra* worked out by W. F. Loomis (1955a, 1955b), was performed in the Loomis Laboratory.

The polarograph, already discussed as applied, registered a decomposition voltage specific for the sulfhydryl group of a thiol compound. The comparative method of polarography identified the recorded substance to be reduced glutathione.

4. *Reagents*

Reduced glutathione as well as all other substances used in the comparative approach—polarographically to identify materials responsible for wave formation—were obtained from the Nutritional Biochemicals Corporation. Triple-distilled mercury was used in all polarographic procedures.

"Pre-purified" nitrogen (Airco) assayed 99.998% and oxygen (Airco) assayed 99.5%. Gases were passed through gas-equilibrated water and directed by capillary pipette into the micro-cell solution chamber. During routine polarographic procedure, nitrogen was passed over the surface of the gut content sample. In certain specified instances oxygen or nitrogen, as the case may have been, was bubbled through the sample *via* a capillary. The latter procedure, when applied, was carried out immediately prior to analysis.

Results

1. *Polarograms recorded immediately following sample isolation from the hind-gut*

Dissolved oxygen in solution is reduced to hydrogen peroxide at the dropping mercury electrode. Kolthoff and Lingane (1952) reported the half-wave potential of the corresponding wave, indicating this reduction is practically independent of pH, at a reading of −0.05 volt *versus* a saturated calomel electrode (S.C.E.).

More than 100 polarograms have been recorded of *Cryptocercus* hind-gut content. The samples ranged in volume from slightly less than 0.1 ml., when one individual roach was used, to approximately one ml. collected from 10–12 roaches. It was impossible to demonstrate an oxygen wave. In particular, ordinary exposure of the gut content to air, incidental to its hasty transfer from insect to the solution chamber of the polarographic cell, failed to introduce a detectable amount of oxygen (Curve A, Figs. 2, 3, 5; Curves A and B, Fig. 6).

Polarograms consistently included a prominent wave at a negative half-wave potential ($-E\ \frac{1}{2}$), ranging between −0.19 to −0.25 v. *vs.* S.C.E. Absence of complete specificity in the $-E\ \frac{1}{2}$ reading suggested the recorded substance within the gut content was organic and pH-dependent. According to Meites (1955), a nonspecific $E\ \frac{1}{2}$ associated with a substance showing polarographic reversibility (as appears within gut content) is more likely attributable to a variable pH than to varying ionic strength in an unaltered medium with regard to buffer.

No direct evidence for the correlation between $-E\ \frac{1}{2}$ and the initial gut content pH for use as a reference point was sought. It was anticipated that delay, in the event sufficient sample remained for polarographic study after pH measurement of a volume as small as 0.1 ml., would be accompanied by sample deterioration. Cell activity gradually changes the chemical nature of hind-gut content as confined metabolites accumulate. The pH of freshly obtained gut content samples ranges between 6.6 and 7.2.

Attempted sample pH control during polarography by oxygen-free buffer addition probably resulted in failure for one or more of the following reasons: (1) sudden medium alteration due to contact with lysed cell content, (2) severe sample dilution, and (3) ionic strength change leading to nonspecificity of $-E\ \frac{1}{2}$ identical to that caused by variable pH for which control was sought ionically.

Slight variation, therefore, in $-E\ \frac{1}{2}$ between polarograms of different freshly obtained gut content samples is attributed to pH variation between samples. Initial standardization of pH, and, for that matter, pH control during a series of recorded polarograms tracing changes produced by *aging* of any one sample as reported later, cannot be achieved successfully with buffer. Regardless, a low oxidation-reduction potential is indicated, and specificity range is sufficiently limited to allow positive identity of the reducing agent.

2. *Tests used in identification of the reducing agent*

A. Polarography

Separate polarograms for solutions of varied substances in 0.1 *M* KCl failed to produce waves even remotely resembling the shape of Curve A, Figure 2, much less a half-wave potential falling within the desired range. Among compounds studied

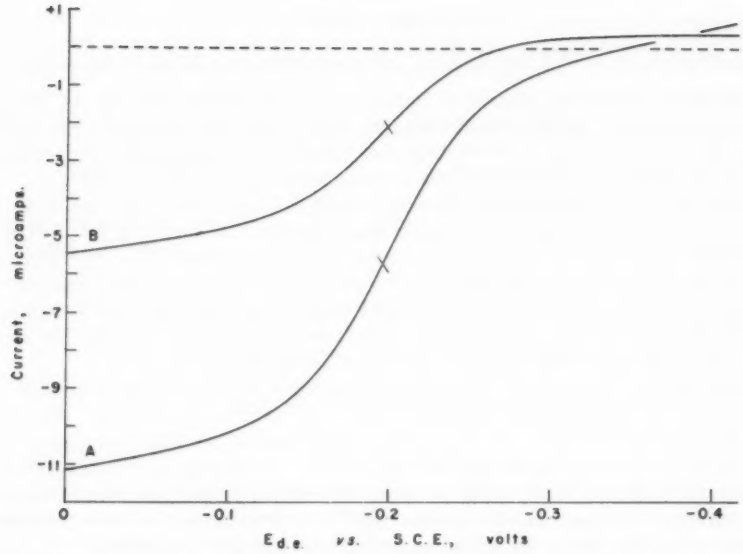

FIGURE 1. Polarograms of reduced glutathione: (A) 1.4×10^{-3} *M* GSH in 0.1 *M* KCl, (B) 7.2×10^{-4} *M* GSH in a complex salt medium also containing yeast, blood serum and methylcellulose (sensitivity X 5; pH 6.6).

were L-cysteine, adrenochrome semicarbazone, ascorbic acid, sodium anthraquinone sulfonate, acetylcholine chloride, riboflavin, and sodium thioglycollate.

A 1.4×10^{-3} *M* solution of reduced glutathione (GSH) in a supporting electrolyte of 0.1 *M* KCl at pH 6.6 produced a polarogram (Curve A, Fig. 1) closely resembling that of Curve A, Figure 2. Addition of 0.005 gm. GSH to a gut content sample increased the wave height without significant change in the previously recorded half-wave potential.

The comparative method of wave height-concentration plots indicated 7.2×10^{-4} *M* GSH in an isotonic medium of mixed salts, nutrients, and methyl cellulose results in a polarogram (Curve B, Fig. 1) almost identical to that recorded from the natural gut content (Curve A, Fig. 2). The Ilkovič equation, mentioned earlier, was not employed in the calculation of GSH concentration. Application of this equation would require a viscosity reading for hind-gut content, and this has not yet been determined.

B. Chemical

GSH produces a violet color in the presence of sodium nitroprusside. A positive reaction occurs when a thin layer of dark brown *Cryptocercus* gut content is tested on a white porcelain surface. Although ascorbic acid does not react with nitroprusside, cysteine and possibly other sulfhydryl compounds effect the positive reaction (Patterson and Lazarow, 1953).

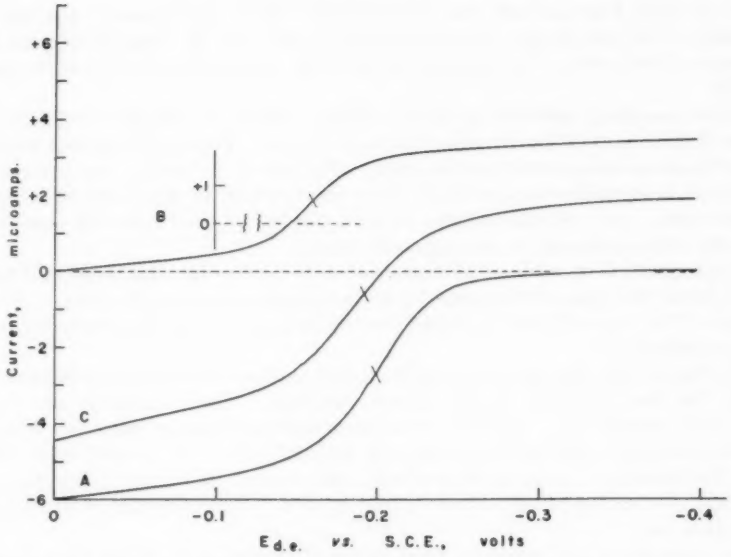

FIGURE 2. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) untreated, (B) following air passage for one hour, and (C) following nitrogen gas flow, 20 minutes (sensitivity X 5; pH approximately 6.8 at start).

Patterson, Lazarow and Levey (1949) report alloxan in excess reacts with the sulhydryl of GSH forming an unknown substance with an absorption spectrum maximum at 305 mμ. By this method 3×10^{-4} *M* GSH has been identified in hind-gut content cleared of cellulose and protozoa. The authors mentioned also report equivalent molecular amounts of cysteine, cysteinylglycine, glutamylcysteine, and ascorbic acid in the presence of GSH lower the value of GSH determination.

C. Bioassay

Loomis (1955a, 1955b) showed that a concentration of 10^{-5} *M* GSH elicits a characteristic feeding response in starved *Hydra littoralis*. A 1:3000 dilution of *Cryptocercus* gut content induced a strong response, indicating a concentration of GSH somewhere in the order of 10^{-3} *M*. This bioassay, however, is no longer specific for GSH. Lenhoff and Bovaird (1960) obtained the same response with trypsin, and Cliffe and Waley (1958) obtained it with ophthalmic acid.

3. *The apparent source of GSH*

Since variables involved with polarograph operation were held constant, slight variations in GSH concentration, observed in fresh unaltered samples, must be inherent in sample fluids. The ratio between number of cells and surrounding fluid volume probably varies between hosts. If GSH is protozoan and/or bacterial in

origin, it could fluctuate with the rise and fall of these populations. If GSH is a secretion of the host tissues, its concentration could vary in direct proportion with the mass of the insect. The question of primary concern, however, was the source of GSH.

After squeezing, sufficient protozoa remain within the roach to repopulate all species back to normal level within four to six weeks. During this period, contrary to conditions in unsqueezed roaches, many cells appear in various stages of mitosis. Protozoan repopulation is a relatively slow process, but the fluid content is rapidly reestablished. An adequate volume of fluid can be removed from the same roach 24 hours after squeezing for polarographic study.

Polarograms were made at 24 hours, and at the end of the first, third, and fourth weeks during the repopulation period with two groups of squeezed roaches. Results indicate GSH concentration increases directly proportional to the increasing protozoan population.

For the present, the relatively crude method used in estimating repopulation rate during the four-six-week period appears adequate. Three stages in the process were easily recognized. (1) That time immediately following squeezing when the gut fluid contains a few random, scattered, residual cells. (2) A mid-stage follows when fluid contains a large number of cells, but also cell-free areas. (3) Maximum population is reached when a mass of cells appears, each in contact at many points with other cells.

An estimate of one week for total bacterial repopulation, following squeezing, exceeds the mean reproductive potential of such organisms. If host tissue was responsible for GSH secretion, a maximum concentration would be expected (polarographic inspection) within one week after squeezing. Usually three weeks were required before GSH concentration reached 50% of normal. This corresponds to a protozoan population judged to be at the approximate refaunation half-way mark.

Several protozoan species are intimately associated with one or more species of bacteria. The cell membrane of *Barbulanympha* shows uniform cellular arrangement for one species of bacterial rod. The association is permanent. This example of *subordinate* symbiosis, and other associations of a similar nature, prevent final decision regarding the exact source of GSH—the protozoa *per se,* intimate bacterial associates, or both. It is, however, significant to know GSH is not host elicited.

4. *The effect of oxygen and nitrogen on GSH*

The relationship between absence of a polarographic oxygen wave, following exposure of gut content to air or pure oxygen, and GSH concentration must be considered.

A sample of hind-gut content bubbled with air at a slow rate for as long as one hour in the H-cell solution chamber failed to produce an oxygen wave. GSH concentration, however, decreased approximately one-half from a reading of approximately 5.4 μa to 2.5 μa shown in a comparison of Curves A and B, Figure 2. The reduction potential also was shifted 0.04 volt more positive.

Roughly 90% of the initial GSH concentration (presumably oxidized) was reduced in the gut content during nitrogen passage for one hour. Recovery was indicated by an increase in the height of the wave (Curve C, Fig. 2) and a reduction

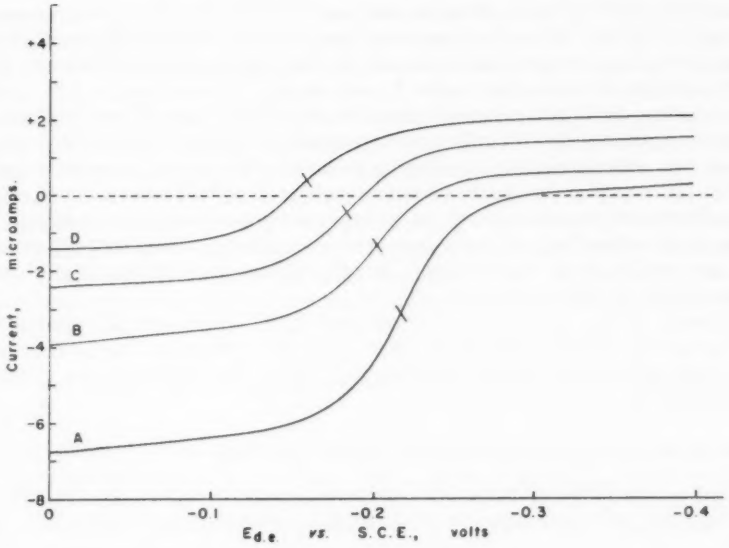

FIGURE 3. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) untreated, (B) following pure oxygen passage ten minutes, (C) following a 20-minute additional period of pure oxygen passage, and (D) nitrogen gas passage, 40 minutes (sensitivity X 5; pH approximately 6.8 at start).

potential shifted back to a more negative reading. Shift of $-E\ ½$ to a more negative position was first viewed as a result of alkalinity increase following a CO_2 *blow-off*. But, upon further consideration, oxygen passage would also be expected to achieve the same effect, and does not. A change in pH, nevertheless, first in one direction and then the other, appears responsible for corresponding shifts in reduction potential.

Curves B and C, Figure 3, show a progressive reduction in GSH concentration for one sample following contact with pure oxygen. Curve A represents the sample's GSH level prior to oxygen exposure. Curve A differs from the previous experiment's unexposed sample (Curve A, Fig. 2), registering approximately 0.7 μa more GSH and a slightly higher pH, judging from the more negative E ½ position.

Curve B, Figure 3, was recorded following oxygen passage for ten minutes. From a comparison of microampere readings between Curve A (original concentration) and Curve B, estimated GSH drop was 50%.

Curve C, Figure 3, was recorded following oxygen passage lasting 20 additional minutes, in which the GSH level was further reduced. Even under these extreme conditions, in a sample less than 0.5 ml., it was not possible to demonstrate the presence of dissolved oxygen.

Continuing with the same sample, nitrogen passage—following oxygen exposure of the extreme intensity just described—failed unaccountably to demonstrate the

reversibility achieved with nitrogen after air exposure in the earlier experiment (Curve C, Fig. 2). Even forty minutes' contact with nitrogen (Curve D, Fig. 3) did not bring about a curve height increase beyond that recorded in Curve C, Figure 3. The height of Curve D, Figure 3, was depressed even further. In addition, again contrary to results obtained when the air-treated sample was bubbled with nitrogen, a comparison of half-wave potentials in Figure 3 illustrates nitrogen passage was accompanied by a reduction potential shift to a more positive reading.

The phenomena just described are based on repeated observations. Polarograms illustrated trace events related to two treated gut samples; one sample using air passage followed by nitrogen gas (results verified by fourteen experiments), and one sample using "prepurified" oxygen followed by nitrogen gas (results verified by seven experiments).

Although it has not been determined how much oxygen a small sample of gut fluid can *absorb* or *bind* without losing its potential with respect to GSH reduction during nitrogen passage, experiments suggest oxygen can overwhelm the glutathione recovery system.

A. Evidence suggesting enzymatic reduction of GSH

That partial GSH reduction can occur during nitrogen passage through a gut sample under prolonged exposure to air suggests strongly the presence of an appro-

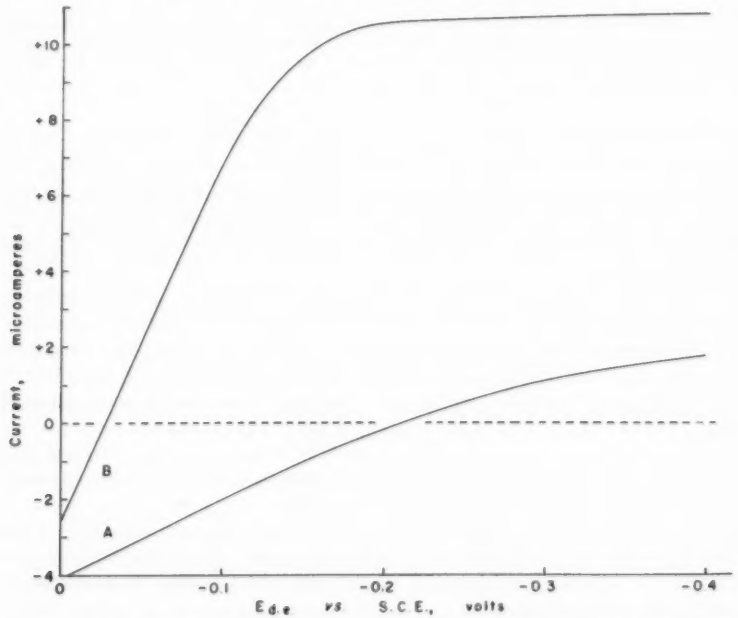

FIGURE 4. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) heated ten minutes, and (B) following pure oxygen passage five minutes (sensitivity X 2).

priate enzyme system. Further evidence favoring the enzyme hypothesis appears when partial glutathione recovery, to the reduced state following air exposure, cannot be achieved in a sample altered by heating over a water bath for 10 minutes at 80° C. Even negligible atmosphere exposure encountered in transferring a heat-treated sample to the electrolysis vessel is sufficient to oxidize GSH, as indicated by disappearance of the reduction wave (Curve A, Fig. 4). Five minutes of oxygen passage then results in such marked saturation of the sample that a prominent oxygen wave (Curve B, Fig. 4) can be recorded.

Glutathione reductase and dehydrogenase assays have not yet been undertaken. Such studies will be resumed following application of present findings to protozoan culture. It is entirely possible that more than one enzyme will be found present in the hind-gut content, accounting for rapid glutathione auto-reduction demonstrated prior to heat alteration.

5. *Evidence suggesting a chemical relationship between glutathione and carbon dioxide*

Polarographic experiments and related observations repeatedly point out another phenomenon in the gut content which might support M. Calvin's (1953) hypothesis for addition of mercaptans across C = O in CO_2 in slightly alkaline media to form S-alkyl monothiocarbonate. He predicted an acid medium could reverse the reaction to release CO_2 and restore GSH:

$$GSH + \underset{\|}{\overset{O}{C}} = O + OH^- \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} R - S - \underset{\|}{\overset{O}{C}} - O^- + H_2O$$

A dense resident population of microorganisms in the hind-gut of *Cryptocercus* ferments ingested cellulose. The fermentation products would be expected to be similar to those reported by Hungate (1939) for *Zootermopsis*—CO_2, hydrogen, and predominantly acetic acid. Complete acid analysis has not been made, but total CO_2 in freshly removed gut content measures 0.12 volume per cent by Scholander's (1947) method.

Fermentation probably continues briefly, following sample introduction into a polarographic vessel (due to continued cell activity), which partially accounts for increase in acidity. Twenty to 30 minutes later the pH drops from the 6.6–7.2 range to 6.4. Within five hours the pH drops to 4.8–5.1, and at 18 hours 4.4. This could explain the shift of half-wave potential in polarograms (Curves A, B, and C, Fig. 5) from −0.196 volt *vs.* S.C.E. made immediately to a more positive potential approximating −0.133 volt after 17 hours.

The protozoa are dead, most are deformed, and some are lysed when the pH reaches 6.4. Motile bacteria are no longer active. The remaining bacterial species constitute a very small number of cells in what was an overwhelmingly populated milieu. These bacterial species can be cultured in a cellulose-nutrient medium which supports growth and reproduction of five protozoan genera normally associated with the bacteria (Ritter, 1959). Bacterial metabolites produced in this culture medium in the absence of protozoa cause the pH to increase, not decrease.

Bacterial influence in this phenomenon of acidification seems to be completely ruled out following observation of toluene-treated gut content samples. Toluene addition to the extent of 20% final dilution failed to alter the pattern of pH decrease to the 4.0–5.0 range within 18 hours.

The cause of pH drop from 6.4 to 4.4 in 18 hours must be accounted for if, as seems to be the case, protozoa and bacteria are not responsible. It appears that bound CO_2 is released in sufficient quantity to cause this pH drop. CO_2 was identified as a major gas constituent, accumulating after several hours as a bubble mound at the interface of a large gut fluid sample layered with heavy mineral oil.

Correlated with this rapid accumulation of CO_2 in the absence of fermentation, polarograms (Curves A and C, Fig. 5) lend additional support to Calvin's hypothesis. As the pH registers progressively lower, GSH concentration increases. From comparative wave height measurement of polarograms A and C, GSH (which almost doubles in quantity within five hours) increases nearly four times the original amount within 17 hours.

This marked wave height increase could indicate mercaptide formation. It is generally accepted that a typical mercaptan characteristic is reaction with heavy metals such as mercury, and with organic compounds, to form mercaptides. And, apparently, there is every opportunity for this reaction to occur in a sample which rests on mercury in the electrolysis vessel.

The mercaptide phenomenon, if it occurs in this gut fluid, does not appear polarographically. The wave height increase represents GSH concentration in-

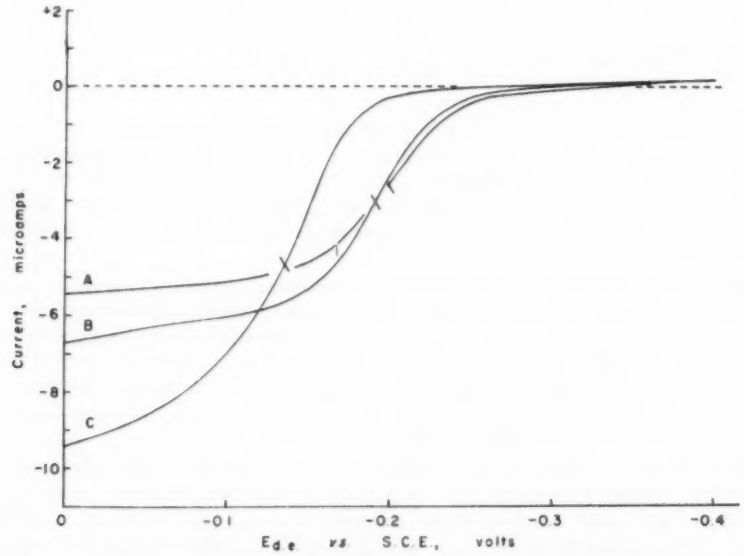

FIGURE 5. Polarograms of the same hind-gut sample from *Cryptocercus*, untreated, and recorded at intervals: (A) immediately, (B) at 20 minutes, and (C) at 17 hours (sensitivity X 5 Curves A and B; X 10 Curve C; pH approximately 6.8 at start; 4.4 at end).

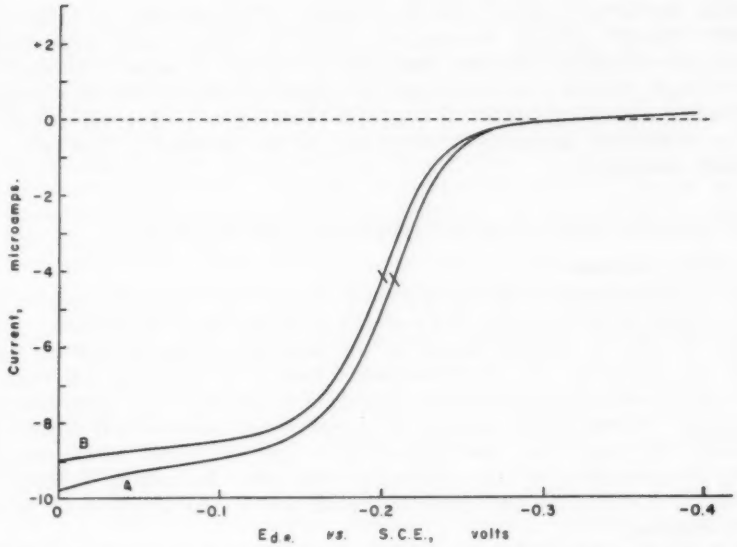

FIGURE 6. Polarograms of two different hind-gut samples from *Cryptocercus:* (A) *aged* 5 hours in the electrolysis vessel and in contact with the mercury pool, pH 5.1, and (B) *aged* 4½ hours in a vessel without mercury contact until transfer to the electrolysis vessel, pH 4.9 (sensitivity X 5).

crease, and the shift in $-E\ \frac{1}{2}$ to a more positive reduction potential correlates with pH drop. Evidence for this conclusion rests on:

1. A fluid sample *aged* between four and five hours in a mercury-free container, after removal from the roach and deposit inside the electrolysis vessel, registers a wave (Curve B, Fig. 6) coresponding closely with a fluid sample *aged* the same period in solution chamber contact with mercury (Curve A, Fig. 6).

2. According to Benesch and Benesch (1952), more energy is required for mercaptide complex reduction than for the free compound that gave rise to the complex. Therefore, the resulting reduction wave would register at a more negative $E\ \frac{1}{2}$ for the mercaptide—not more positive as recorded in this study—to reflect the low dissociation of the Hg-S bond.

3. Also in the event of mercaptide formation, two separate waves would be expected to appear polarographically. The difference between the $-E\ \frac{1}{2}$ of the gradually forming mercaptide and the more negative $E\ \frac{1}{2}$ of disappearing GSH would likely be great enough to initiate two separate reduction waves separated by a shifting plateau.

DISCUSSION

The appreciable presence of atmospheric oxygen coupled with diverse, unique, and efficient animal systems that transport oxygen suggest unspecified claims of anaerobiosis (as to degree) should be questioned. Living membranes present an

ineffectual barrier to oxygen. For that matter, full appreciation of oxygen permeability through synthetic membranes cannot be realized until experience is acquired with a *working* chamber from which all oxygen is meant to be excluded. In other words, at least a minute amount of oxygen probably reaches an organism's most remote anatomical niche. If symbionts dwelling in such a site require an absolute oxygen-free environment, either they or the surrounding host cells must chemically maintain it.

1. *Absence of molecular oxygen in* Cryptocercus *hind-gut content*

A. Direct evidence

The entire problem of determining environmental pO_2 at lower limits is hampered by inadequate indicators and mechanical devices for measurement. Polarography, in spite of extreme sensitivity for detecting oxygen in solution, is incapable of registering ultra-trace amounts. Failure to obtain typical reduction waves for oxygen in *Cryptocercus* hind-gut fluid polarograms is of great analytical significance. However, the question of degree in anaerobiosis still remains unanswered. To report that this environment is free of oxygen to the extent of 10^{-6} *M* is informative, though admittedly inadequate, for culture investigation. Technically, therefore, the term anaerobic should not be used merely on the basis of this information.

B. Indirect evidence

The polarograph also indicated concentration decrease of an unknown substance when gut content was exposed to pure oxygen. Prolonged oxygen exposure failed to leave enough molecular oxygen in solution for polarograph detection. In addition to indicating a substance is exploited in proportion to the amount of oxygen passing into gut fluid, the observation strongly suggests chemical affinity between oxygen and this unknown substance.

Oxygen most certainly diffuses into the lumen from the gut epithelium under natural conditions. Conceivably the oxygen is instantly bound molecularly. Interpretation of these phenomena, based on direct and indirect evidence obtained in polarograms, suggests *Cryptocercus* protozoa live in an anaerobic environment—anaerobic to the extent of maximum physical and chemical maintenance.

2. *Reduced glutathione in* Cryptocercus *hind-gut content*

A. Methodology in identification

Identification of the substance, which registers so prominently on polarograms and appears to function as a strong reducing agent, was difficult. Analyses offering unequivocal interpretation are rare when attempting to single out one organic constituent in the complexity of a biological medium.

Investigative methods included comparative polarography, the classic sodium nitroprusside test, the alloxan "305" method, and the Loomis bioassay technique.

Comparative polarography is a highly accurate procedure in which closely controlled physical conditions are imposed to measure known and unknown solutions. A more extensive survey of potential reducing agents appeared unnecessary because of the method's relative specificity. A known GSH solution produces

a polarogram of nearly identical structure to gut content polarograms. The supporting electrolyte of mixed salts used in the GSH medium was determined by flame photometry of the gut fluid lacking major suspended elements. Viscosity was approximated with methyl cellulose, and the pH of 6.8 required no adjustment. Variation in any one of these—especially pH—caused an expected fluctuation in the $-E\ \frac{1}{2}$, but the polarographic curve remained essentially unchanged.

As mentioned earlier, the standard addition method of polarography confirmed the presence of glutathione. Addition of GSH to the unknown sample increased the wave height (concentration) without changing other aspects of the curve.

Following preliminary evaluation of other available methods of GSH analysis for application to this study, one-dimensional paper chromatography—of use after blocking the sulfhydryl group with N-ethylmaleimide (Hanes, Hird and Isherwood, 1950)—indicated greatest specificity. Inadequate sensitivity of the chromatographic method, using GSH concentration determined polarographically as reference, eliminated chromatographic application.

The alloxan "305" method functioned as a qualitative test only. GSH concentration determined in the supernatant portion of centrifuged gut content was less than half the amount demonstrated polarographically. Although substances already mentioned compete with GSH in alloxan reaction, it is possible only two need be considered. Cysteine and ascorbic acid, though potentially reactive at the dropping mercury electrode, do not appear polarographically. Glutamylcysteine and/or cysteinylglycine might be involved in competitive absorption. Awaiting further investigation, one might guess that γ glutamylcysteine, an intermediate in GSH synthesis probably to the exclusion of cysteinylglycine (Snoke and Bloch, 1952), is of more vital concern in evaluating potential alloxan competition with GSH.

The nitroprusside method and the bioassay technique using *Hydra* lack specificity in GSH identification. Nevertheless, nitroprusside identified the substance as a sulfhydryl compound, and *Hydra* gives a feeding response only in the presence of reduced glutathione. Trypsin, as yet unidentified in *Cryptocercus,* has not been ruled out as an alternative stimulant for *Hydra.*

This represents by no means an exhaustive methodology for assaying biologically-occurring glutathione. Each chemical test available is nonspecific or limited to some degree. General disagreement usually exists regarding the number of chemical tests necessary before identification is scientifically acceptable. In this polarographic study, however, more than mere substantial evidence accumulated to support glutathione assessment. Results derived from both nitroprusside and *Hydra* assays were conclusive yet predicated upon far less specific analysis.

On the strength of collective information afforded by these tests, along with confirming opinions of Professors K. E. Bloch, J. T. Edsall, and A. M. Pappenheimer, GSH is a constituent of *Cryptocercus* hind-gut content. GSH is responsible for maintenance of the oxidation-reduction potential observed polarographically.

B. The site of synthesis

Since more than 30 species of protozoa inhabit *Cryptocercus,* it must still be determined which of these are involved in GSH synthesis. The problem is further complicated by intimate, and, in some cases, permanent, bacterial association with the cell membrane of certain protozoan species. Evidence suggests protozoa are the site for this synthesis, but not necessarily the sole metabolic source of GSH.

C. Oxidation-reduction activity

It was impossible to assess fully results obtained in experiments in which gas was directed through freshly obtained gut content samples. It was clear that oxygen decreased the concentration of reduced glutathione. This was interpreted as an oxidative phenomenon in which GSH was converted to the disulfide form (GSSG). Although nitrogen passage was capable of partially restoring the glutathione wave height in air-exposed samples, the precise mechanism was not discernible. It has been assumed temporarily that this wave extension represents a reduction of GSSG to GSH—the nitrogen gas being most indirectly involved.

Wieland (1953) stated reduction of GSSG can be accomplished with various reducing agents, other mercaptans in excess, nascent hydrogen, Na or Li in NH_3, and by other substances. He also pointed out enzymatic reduction is frequently responsible. Whatever the cause, a point exists—polarographically demonstrated—at which nitrogen can no longer carry out its indirect role. This occurs following gut content exposure to pure oxygen in excess of ten minutes. Glutathione oxidized to this extent could not be *reactivated* as could samples exposed to lesser amounts of oxygen.

Opinions sought are totally inadequate in accounting for contradictory evidence concerning the effect of nitrogen following varied aerations of gut content—air *versus* pure oxygen. Interpretation of present phenomena obtained polarographically must await correlation with results from additional analyses now contemplated.

Since the oxidation-reduction potential of gut content can also be rendered inactive by heat, the tentative conclusion for the presence of at least one enzyme system seems justified. Glutathione reductase, according to Vennesland and Conn (1953), is the thermolabile enzyme responsible for the reduction of GSSG by tissues. GSSG is reactive with TPN-reducing systems. Accordingly: $TPNH + GSSG + H^+ \rightarrow TPN^+ + 2GSH$

D. A proposed role in pCO_2 and pH regulation

Culture studies in progress demonstrate glutathione can now be assigned an extracellular role in anaerobic maintenance. The importance of this mercaptan in growth and other physiological activities is well substantiated, but whether it can be assigned an additional function in the gut of *Cryptocercus* is still problematical.

Speculation regarding GSH capacity to combine with CO_2 to form an alkyl compound, as proposed by Calvin, is a tempting hypothesis. Culture studies of the protozoa definitely show a very narrow range of pCO_2 tolerance. Experimentally, membrane selectivity can be altered dramatically when cells are exposed to an excess of this gas, but protozoa move directly to a higher pCO_2 gradient when placed in a sub-optimal region of the culture medium.

Hind-gut protozoa, therefore, engaged in active fermentation yet highly sensitive to an altered pCO_2 threshold, would have marked survival potential if a mechanism were available for chemical regulation of this metabolite. Theoretically GSH could function as a pH-regulated pCO_2 buffer, supplementary to more direct pCO_2 control by the roach's external respiratory apparatus. Conceivably, even though pH might *trigger* this mechanism to reestablish an equilibrium between S-alkyl monothiocarbonate and GSH, pH, in turn, could be regulated, in part at least, by the mechanism. Certainly the host roach and its protozoa are confronted

by environmental factors that challenge optimal metabolism, such as conditions imposed during molting and winter.

I am very much indebted to Dr. L. R. Cleveland for invitation to work at the Biological Laboratories, for his advice, and for his criticism of the manuscript. I am grateful for the assistance of Dr. W. F. Loomis, and for advice and suggestions regarding polarography and the interpretation of polarograms from Professor J. J. Lingane and Dr. Allen J. Bard. Professors Bloch, Edsall, Pappenheimer, and Wald were consulted during various phases of this study. Their interest and counsel is greatly appreciated.

Summary

1. Polarography, employing a mercury-dropping electrode and an electrolysis vessel for analysis of sample slightly less than 0.1 ml., was applied in this study.

2. Polarograms of *Cryptocercus* hind-gut content lack an oxygen wave and indicate absence of dissolved oxygen to the extent of apparatus sensitivity (10^{-6} M).

3. A single polarographic wave registers a half-wave potential approximating −0.2 volt *vs.* S.C.E. within a 0- to −0.4-volt span. Specificity of this method enabled identification of reduced glutathione (7.2×10^{-4} M) following comparative inspection of numerous reducing agents. Supplementary assays using sodium nitroprusside, alloxan monohydrate, and *Hydra* confirmed the presence of reduced glutathione in hind-gut content.

4. Flagellate protozoa, symbionts in the hind-gut fluid of *Cryptocercus*, represent the site of glutathione synthesis.

5. Prolonged oxygen passage through hind-gut samples (0.1–0.5 ml.) fails to alter polarograms with respect to negative pO_2 registration, but reduced glutathione concentration is decreased in direct proportion to oxygen exposure intensity.

6. Partial restoration of reduced glutathione concentration following nitrogen gas passage through samples moderately exposed to oxygen cannot be explained.

7. Brief atmosphere contact with heat-altered hind-gut samples results in polarograms showing pO_2 contamination and an absence of reduced glutathione. Until enzyme analyses are completed, sample alteration by heat merely suggests presence of a mechanism for glutathione maintenance in the reduced state.

8. *Cryptocercus* hind-gut content is anaerobic to the extent of maximal physical and chemical maintenance as are the microorganisms normally living within it. Reduced glutathione is considered of major importance in this role.

9. Some evidence exists which may support Calvin's hypothesis for addition of mercaptans across carbonyls in a biological sample. The value of such a mechanism to *Cryptocercus* and its symbionts is theorized.

Literature Cited

Beecher, H. K., R. Follansbee, A. J. Murphy and F. N. Craig, 1942. Determination of the oxygen content of small quantities of body fluids by polarographic analysis. *J. Biol. Chem.*, **146**: 197–206.

Benesch, R., and R. E. Benesch, 1952. Reactions of thiols with organic mercury compounds. *Arch. Biochem. and Biophys.*, **38**: 425–441.

Benesch, R., R. E. Benesch and W. I. Rogers, 1953. The reactivity of the sulfhydryl group in glutathione and related peptides. *In:* Glutathione, 1954 (S. Colowick *et al.*, editors), Academic Press, Inc., New York: 31–42.

CALVIN, M., 1953. Mercaptans and disulfides: some physics, chemistry, and speculation. *In:* Glutathione, 1954 (S. Colowick *et al.*, editors), Academic Press, Inc., New York: 3–30.

CLEVELAND, L. R., 1925a. The effects of oxygenation and starvation on the symbiosis between the termite *Termopsis*, and its intestinal flagellates. *Biol. Bull.*, **48**: 309–326.

CLEVELAND, L. R., 1952b. Toxicity of oxygen for protozoa *in vivo* and *in vitro:* animals defaunated without injury. *Biol. Bull.*, **48**: 455–468.

CLEVELAND, L. R., S. R. HALL, E. P. SANDERS AND J. COLLIER, 1934. The wood-feeding roach *Cryptocercus*, its protozoa, and the symbiosis between protozoa and roach. *Mem. Am. Acad. Arts and Sci.*, **17**: 185–342.

CLEVELAND, L. R., AND A. W. BURKE, 1956. Effects of temperature and tension on oxygen toxicity for the protozoa of *Cryptocercus*. *J. Protozool.*, **3**: 74–77.

CLIFFE, E. E., AND S. G. WALEY, 1958. Effect of analogues of glutathione on the feeding reaction of *Hydra*. *Nature*, **182**: 804–805.

GILMOUR, D., 1940. The anaerobic gaseous metabolism of the termite, *Zootermopsis nevadensis* Hagen. *J. Cell. Comp. Physiol.*, **15**: 331–342.

GRUNERT, R. R. AND P. H. PHILLIPS, 1951. A modification of the nitroprusside method of analysis for glutathione. *Arch. Biochem. and Biophys.*, **30**: 217–225.

HANES, C. S., F. R. HIRD AND F. A. ISHERWOOD, 1950. Synthesis of peptides in enzymic reactions involving glutathione. *Nature*, **166**: 288–292.

HUNGATE, R. E., 1939. Experiments on the nutrition of *Zootermopsis*. III. The anaerobic carbohydrate dissimilation by the intestinal protozoa. *Ecology*, **20**: 230–245.

KOLTHOFF, I. M., AND J. J. LINGANE, 1952. Polarography. Second ed. Interscience Publishers, Inc., New York.

LENHOFF, H. M., AND J. BOVAIRD, 1960. Enzymatic activation of a hormone-like response in *Hydra* by proteases. *Nature*, **187**: 671–673.

LOOMIS, W. F., 1955a. Specific qualitative microbioassay for reduced glutathione. *Fed. Proc.*, **14**: 247.

LOOMIS, W. F., 1955b. Glutathione control of the specific feeding reactions of *Hydra*. *Ann. N. Y. Acad. Sci.*, **62**: 211–227.

LOWRY, O. H., AND O. A. BESSEY, 1946. The adaptation of the Beckman spectrophotometer to measurements on minute quantities of biological materials. *J. Biol. Chem.*, **163**: 633–639.

MEITES, L., 1955. Polarographic Techniques. Interscience Publishers, Inc., New York.

PATTERSON, J. W., A. LAZAROW AND S. LEVEY, 1949. Reactions of alloxan and dialuric acid with the sulfhydryl group. *J. Biol. Chem.*, **177**: 197–204.

PATTERSON, J. W., AND A. LAZAROW, 1953. Methods of glutathione assay: a review of classical methods. *In:* Glutathione, 1954 (S. Colowick *et al.*, editors), Academic Press, Inc., New York: 63–78.

RITTER, H. T. M., 1959. Factors essential for *in vitro* reproduction of *Cryptocercus* protozoa. *J. Protozool.*, **6** (August supplement): 21.

SCHOLANDER, P. F., 1947. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. Biol. Chem.*, **167**: 235–250.

SNOKE, J. E., AND K. BLOCH, 1952. Formation and utilization of γ-glutamylcysteine in glutathione synthesis. *J. Biol. Chem.*, **199**: 407–414.

TRAGER, W., 1933. The cultivation of some intestinal flagellates of termites and the nature of the symbiosis between these protozoa and their insect host. Thesis, Harvard University.

VENNESLAND, B. AND E. E. CONN, 1953. The enzymatic oxidation and reduction of glutathione. *In:* Glutathione, 1954 (S. Colowick *et al.*, editors), Acadameic Press, Inc., New York: 105–127.

WIELAND, T., 1953. Chemistry and properties of glutathione. *In:* Glutathione, 1954 (S. Colowick *et al.*, editors), Academic Press, Inc., New York: 45–57.

YAMASAKI, M., 1931. Studies on the intestinal protozoa of termites II. Oxygenation experiments under the influence of temperature. *Mem. Col. Sci., Kyoto Imp. Univ., Series B*, **7**: 179–188.

COBALT AND GLUTATHIONE IN THE PRESERVATION OF FERTILITY AND LIFE OF SAND DOLLAR EGGS[1]

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The loss of fertility of the unfertilized egg of *Dendraster excentricus* within a few hours after removal from the ovary is of interest. During the breeding season the eggs may be 95–100% fertilizable for 24–48 hours when allowed to stand uncrowded in finger bowls of sea water at 18° C. After this interval, the capacity for fertilization decreases and the eggs soon disintegrate. When conditions are not optimum the eggs show poor fertility from the beginning. This is commonly the case with over-ripe eggs. The decay of fertility seems to be correlated with the onset and progression of the death processes which lead to disintegration.

Fertility may be prolonged or regained under certain conditions. In experiments involving the treatment of *Dendraster* eggs with lithium before fertilization (Rulon, 1946) it was found that a 1% ethyl alcohol solution would insure the fertility of the egg sufficiently to permit an adequate period of exposure to the test agent. In other experiments (Rulon, 1948) it was found that certain lots of *Arbacia* eggs, which were only 25–50% fertilizable, could be stimulated to 90–100% fertilization by treatment with Ca-free sea water for eight hours before the addition of a few drops of sperm suspension.

For a number of years the writer has been interested in modifying and controlling developmental patterns in echinoderm embryos with various enzyme inhibitors and other agents. In several cases interesting modifications have been caused by treatment of the unfertilized egg (Rulon, 1941, 1946). Cobaltous chloride was chosen for the present study because previous work (Rulon, 1956) had shown it to alter development in a definite manner when applied to the newly fertilized egg and the mid-blastula. In the work reported here it was found that cobalt had little effect on the pattern of the developing larva when only the unfertilized egg was treated. It was found, however, that cobalt possessed remarkable properties for maintaining fertility long beyond the normal period.

Materials and Methods

The sand dollars (*D. excentricus*) were dredged from Monterey Bay during the summer of 1959 and maintained in healthy condition in tanks in the aquarium room of the Hopkins Marine Station, Pacific Grove, California. Ova were taken in large numbers from ripe females by cutting away the oral surfaces of the tests and allowing the exposed gonads to shed. Droplets of ova were drawn into medi-

[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, Director of the Hopkins Marine Station, Pacific Grove, California.

cine droppers and released in fresh sea water. After washing, the ova were placed in the various concentrations of cobaltous chloride and cobaltous-glutathione solutions for the duration of the experiments. At 24-hour intervals a number (50–100) of the eggs were removed to fresh sea water and washed. Fresh sperm suspension was added to test fertility. The chief criterion for fertilization was nuclear division. Throughout the work the eggs were kept uncrowded in covered fingerbowls out of direct sunlight and at 18± 1° C. Smoking was not permitted in the laboratory since previous work by Child and others has shown that in sufficient density, tobacco smoke may cause modifications in developing embryos (Child, 1941, p. 222).

Experimental

I. *The effects of cobaltous chloride on fertility and cleavage*

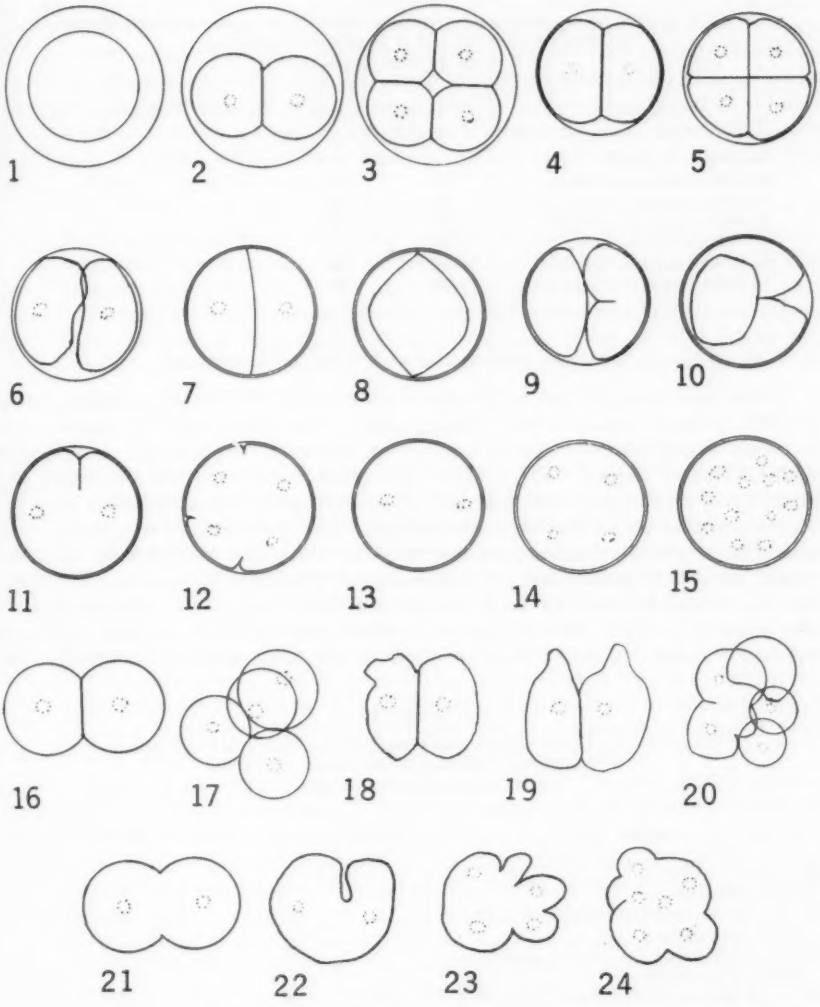
Numerous preliminary experiments indicated unfertilized eggs, in sea-water solutions of cobaltous chloride, retain their fertility long beyond those in sea-water controls. They also showed that on fertilization the cobalt-treated eggs behaved somewhat differently from the controls and that these differences were related to concentration of the cobalt and length of exposure period.

One of the most conspicuous effects of exposure to cobaltous chloride was the presence of *tight* fertilization membranes on fertilized eggs from the more concentrated solutions (compare controls, Figures 1–3, with tests, Figures 4–15). Eggs dividing in such membranes gave elongated and often incompletely separated blastomeres. If the exposure was of sufficient duration cytoplasmic division was partially or totally inhibited but nuclear division still occurred (as in Figures 11–15). If the concentration of cobalt was somewhat less but the exposure time was of long duration, the presence of a fertilization membrane could not be detected at all and cytoplasmic division graded from complete to partial (Figs. 16–24). When the blastomeres were fully cleaved they tended to become spherical and loosely attached. With long treatment many inequalities in size of cells and irregularities in cleavage patterns were to be noted.

It was soon evident that cobalt was exerting its effects largely on the cell cortex and that cytoplasmic division was more subject to inhibition than nuclear division. In general, it was found that most of the eggs that could be fertilized showed recovery in sea water and developed into plutei that were not far from normal. In some cases, however, plutei were somewhat stunted and granular but none developed in a manner comparable to those caused by post-fertilization treatment (see Rulon, 1956).

The effects of various concentrations of cobalt on the preservation of fertility are shown in Table I. Here are tabulated the results of a single series from eggs taken from one female. In this series, approximately 45% of the control eggs were fertilizable at the end of 24 hours (not in table) but none could be fertilized at 48 hours. Approximately 50% responded after treatment with $M/400$–$M/800$ for 96 hours while approximately 25% retained fertilizability for as long as 168 hours with $M/1600$–$M/3200$. Even in a weak solution of cobaltous chloride ($M/6400$) 50% of the eggs retained fertility for 96 hours.

These experiments definitely showed that certain concentrations of cobalt prolong the fertile life and even life itself of the egg of *Dendraster excentricus*.



FIGURES 1–24. Figure 1, newly fertilized egg of *Dendraster* with elevated fertilization membrane. Figures 2–3, normal early cleavage within fertilization membrane. Figures 4–10, eggs cleaving within tight fertilization membrane following exposure to strong (*M*/400–*M*/1600) solutions of cobalt. Figures 11–15, eggs with nuclear division but inhibited cytoplasmic division following long exposure to cobalt. Figures 16–24, eggs with aberrant cytoplasmic division and undetectable fertilization membranes following exposure to less concentrated solutions of cobalt.

TABLE I

The effects of different concentrations of cobaltous chloride for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

Solution used	48 hours	96 hours	144 hours	168 hours	192 hours
Sea water (control)	—	—	—	—	—
M/200 cobaltous chloride	20	10	—	—	—
M/400 cobaltous chloride	50	60	1 –	—	—
M/800 cobaltous chloride	60	40	1 –	—	—
M/1600 cobaltous chloride	80	50	40	30	1 –
M/3200 cobaltous chloride	80	50	25	25	—
M/6400 cobaltous chloride	80	50	—	—	—

II. *The effects of cobaltous glutathione on fertility and cleavage*

It has been thought that cobalt causes much of its effect by the fixation of thiol (−SH) groups (see Marston, 1952). Zinc, which has much the same action as cobalt on differentiation but at much lower concentration, appears, also, to unite with sulfhydryl (Rulon, 1955). Since the effect of zinc may be prevented with glutathione, it was suggested that this agent may well inhibit the effect of cobalt on the preservation of fertility. Accordingly, tests were run in which eggs were placed in solutions of cobaltous-glutathione in which the concentration of cobalt varied but that of glutathione was kept constant at 0.025% (a concentration which had no detectable effect by itself on the unfertilized egg). It seemed apparent that there was actual chemical union of these two substances in that clear pink solution of cobalt becomes a clear brown when the colorless glutathione was added.

TABLE II

The effects of cobaltous chloride and glutathione for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

Solution used	48 hours	96 hours	168 hours	216 hours	312 hours
Sea water (control)	—	—	—	—	—
0.025% glutathione	—	—	—	—	—
50 cc. *M*/100 cobaltous chloride plus 50 cc. 0.05% glutathione	95	95	95	90	—
50 cc. *M*/200 cobaltous chloride plus 50 cc. 0.05% glutathione	95	95	90	70	50*
50 cc. *M*/400 cobaltous chloride plus 50 cc. 0.05% glutathione	95	90	75	75	—
50 cc. *M*/800 cobaltous chloride plus 50 cc. 0.05% glutathione	90	—	—	—	—
50 cc. *M*/1600 cobaltous chloride plus 50 cc. 0.05% glutathione	—	—	—	—	—
50 cc. *M*/3200 cobaltous chloride plus 50 cc. 0.05% glutathione	—	—	—	—	—

* Different series of experiments.

The results of this series of experiments are tabulated in Table II where it is shown that glutathione prevented the fertility-preserving effects of cobalt only at the lower concentrations. In the stronger solutions (50 cc. *M*/100–*M*/400 cobaltous chloride plus 50 cc. of 0.05% glutathione) cobaltous-glutathione was a far more effective agent in prolonging the fertile life of the eggs of *Dendraster* than was cobalt alone. It will be noted that the eggs are still highly fertile after 216 hours in these solutions and that in another experiment there was 50% fertility after 13 days.

It was of interest to note (data not shown in table) that while with the most concentrated solution (50 cc. of *M*/100 cobalt plus 50 cc. of 0.05% glutathione) 90% of the eggs were fertile, only nuclear division resulted. In treatment with next strongest solution, where 70% of the eggs were fertile, 30% showed only nuclear division while the remaining 40% showed some cytoplasmic cleavage as well. The 75% fertile eggs from the next solution (50 cc. *M*/400 cobalt plus 50 cc. of 0.05% glutathione) showed cytoplasmic along with nuclear division.

Discussion

It does not seem likely that the effects of cobalt in the concentrations used in these experiments were due to osmotic factors. Although measurements of egg size were not made, the test eggs, before fertilization, appeared little different from the controls. In previous work by the author and many others, agents far more concentrated (*i.e.*, lithium) have been used with only slight osmotic effects. Minor changes (of the order of the concentrations used in these experiments) in salt content of the sea water produce little or no effects. It is known, however, that as echinoderm eggs age there is an increase in permeability and viscosity (see Goldforb, Landowne and Schechter, 1937, for references). It is not known that such physical changes destroy fertility in the egg but there seems to be a correlation. It should be remembered, however, that permeability to water and ions is quite different from permeability to (or penetration by) spermatozoa.

The failure of the fertilization membrane to become elevated after treatment with cobalt is difficult to explain. Tight membranes occur under a variety of conditions in these forms (*i.e.*, aging itself will cause them). It may be that long exposure to cobalt partially denatures the vitelline membrane or it may be that cobalt prevents the breakdown of the cortical granules. Certainly the subject deserves further study.

Any explanation of the mechanisms for the increase or preservation of fertility is not easy. To this worker it long has been a question as to why an ovum, containing so much in the way of nutrients, should lose its capacity to be fertilized so early. Other investigators have dealt with the problem in other eggs. The fertile life of *Arbacia* eggs may be prolonged in low-calcium sea water (Schechter, 1937). Perhaps such procedure prevents the release of calcium from its protein binding in the cortex for internal clotting (Heilbrunn, 1943, pp. 88–89) and thereby prevents or slows down processes concerned with loss of fertility and death. The fertilizable life of *Urechis* eggs may be extended with 1% alcohol or dextrose (Whitaker, 1937). It was suggested that these agents are effective by furnishing nourishment to the egg or by decreasing permeability of the egg surface. Runn-

ström (1952) reports the use of various amino acids and proteins to improve fertilization (but not to preserve fertility) in echinoderm eggs.

Of the several hypotheses given in the past it seems that the present work with cobalt may lend itself to an interpretation that is similar, in part, to one proposed by Loeb (1912, p. 155) who found that the fertilizable life of the starfish egg could be extended by cyanide or oxygen lack. He concluded that the egg becomes unfertilizable, in the normal course of events, after a damaging amount of aerobic oxidation takes place and that such agents as potassium cyanide preserved fertility by slowing down this action.

The writer has come to a tentative explanation as to the action of cobalt. This substance is well known because of its action in inducing polycythemia, possibly as the result of physiological compensation to partial anoxia, caused by the fixation of −SH groups (see Marston, 1952). It is suggested that the cortex and surface of the newly shed egg are abundant in such groupings. It is well known that essential −SH groups on an enzyme may be rapidly oxidized by atmospheric oxygen (see Singer, 1945). It seems not unreasonable to assume that under normal aerobic conditions in sea water, labile or soluble sulfhydryl compounds may be expected to unite with each other by oxidation to disulfide. In other words, simple proteins are united into large stable, insoluble protein chains in which the union of the individual members is through −S−S− bonding (see Jensen, 1959). Such a change at the egg surface may well account for the loss in fertility in aging eggs.

It is believed that cobalt prevents this chain reaction by uniting with −SH and in this manner serves in preventing the decay of fertilizability. This, indeed, seems to be the case as is shown in the experiments in which glutathione is added to the cobaltous chloride solutions. Concentrations of cobalt that were highly effective in prolonging fertility ($M/1600$–$M/3200$) were rendered partly or entirely ineffective by the addition of 0.025% glutathione. This shows the affinity of the two substances and supports the suggestion that cobalt unites with thiol groupings at the egg surface in the preservation of its integrity. But with more concentrated solutions of cobaltous-glutathione *the preservation of fertility was greatly enhanced!*

It is apparent that a different situation has now developed. The combination gives more protection to the egg surface and cortex than does the cobaltous ion alone. In the adsorption of cobaltous-glutathione, changes (oxidation of −SH) with the loss of fertility are considerably less than with cobalt alone. Such an explanation seems highly speculative until one considers the more recent work with Co^{60} Vit. B_{12} in which the combining power of this important substance with the serum proteins (alpha and beta globulins) and the protein of cerebro-spinal fluid has been so aptly demonstrated (see Meyer *et al.*, 1959). It seems quite reasonable to assume that the remarkable preserving action of cobaltous-glutathione is brought about by its union with the substances which would otherwise gradually change as fertilizability is lost.

Summary

1. A range ($M/200$–$M/6400$) of cobaltous chloride in sea water increases the fertile life of *Dendraster* eggs. The most effective concentrations ($M/1600$–

$M/3200$) prolong fertilizability for over 168 hours in 25–30% of the eggs tested. None of the control eggs is fertile at 48 hours.

2. The exposure of unfertilized eggs for long periods or to high concentrations leads to tight fertilization membranes and gradual suppression of cytoplasmic division when the eggs are finally fertilized.

3. Glutathione (0.025%) prevented the fertility-preserving action of cobaltous chloride in lower concentrations but greatly enhanced it in higher concentrations. One mixture (50 cc. of 0.05% glutathione plus 50 cc. $M/200$ cobaltous chloride) preserved fertility in 50% of the eggs for 312 hours.

4. The work suggests that cobalt and cobaltous-glutathione unite with R–SH at the egg surface (each in a somewhat different manner) preventing oxidation and a deterioration of fertilizability.

LITERATURE CITED

CHILD, C. M., 1941. Patterns and Problems of Development. Chicago: University of Chicago Press.

GOLDFORB, A. J., M. LANDOWNE AND V. SCHECHTER, 1937. Effects of age on *Arbacia* eggs. VI. Increased stretching and bursting of egg membrane in hypotonic sea water. *Physiol. Zoöl.*, **10**: 59–70.

HEILBRUNN, L. V., 1943. An Outline of General Physiology. Second Edition. Saunders, Philadelphia.

JENSEN, E. V., 1959. Sulfhydryl-disulphide interchange. *Science*, **130**: 1319–1323.

LOEB, J., 1912. The Mechanistic Conception of Life. University of Chicago Press.

MARSTON, H. R., 1952. Cobalt, copper and molybdenum in the nutrition of animals and plants. *Physiol. Rev.*, **32**: 66–121.

MEYER, L. M., R. W. BERTCHER AND C. MULZAC, 1959. $Co.^{60}$ Vit. B_{12} binding capacity of normal human cerebrospinal fluid. *Proc. Soc. Exp. Biol. Med.*, **100**: 607–608.

RULON, O., 1941. Modification of development in the sand dollar by NaCNS and Ca-free sea water. *Physiol. Zoöl.*, **14**: 305–315.

RULON, O., 1946. Modification of sand dollar development by exposure to lithium chloride and sodium thiocyanate before and after fertilization. *Physiol. Zoöl.*, **19**: 58–66.

RULON, O., 1948. The experimental modification of developmental patterns in *Arbacia* with malonic acid. *Physiol. Zoöl.*, **21**: 100–105.

RULON, O., 1955. Developmental modifications in the sand dollar caused by zinc chloride and prevented by glutathione. *Biol. Bull.*, **109**: 316–327.

RULON, O., 1956. Effects of cobaltous chloride on development in the sand dollar. *Physiol. Zoöl.*, **29**: 51–63.

RUNNSTRÖM, J., 1952. The cell surface and its relation to fertilization. Symposia of the Society for Experimental Biology, VI: 39–88. University Press, Cambridge.

SCHECHTER, V., 1937. Calcium reduction and the prolongation of life in the egg cells of *Arbacia punctulata*. *Biol. Bull.*, **72**: 366–376.

SINGER, T. P., 1945. Enzyme inhibitors and the active groups of proteins. *Brewers' Digest*, **20**: 43–46 (August), 42–44 (September).

WHITAKER, D. M., 1937. Extension of the fertilizable life of unfertilized *Urechis* eggs by alcohol and by dextrose. *J. Exp. Zool.*, **75**: 155–167.

LEARNING IN THE SPINY LOBSTER PANULIRUS ARGUS

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Although Bethe (1898) believed crustaceans in general could not learn by experience, subsequent research by many investigators has proved that cladocerans, isopods, crayfishes, hermit crabs and several kinds of brachyuran crabs can variously show habituation, discrimination learning and trial-and-error learning (reviews: Warden, Jenkins and Warner, 1940; Thorpe, 1956; Schöne, 1961; recent research paper: Datta, Milstein and Bitterman, 1960). In these different cases visual, chemoreceptor, kinesthetic or tactile elements were involved in the afferent components of the behavior patterns studied. As is typical for learning, these data (plotted as error scores) usually fall on curves resembling exponential decay functions with a large initial number of errors which, with training, are rapidly reduced at first and then later decrease more and more slowly (Hull, 1952; Hilgard, 1956; Bush and Estes, 1959).

In attempting to demonstrate polarized light vision in decapod crustaceans, training experiments were undertaken with *Panulirus* to determine whether different planes of polarization could be learned as cues for the solution of a two-choice spatial problem. While the lobsters failed to use the polarization pattern in this way, they were able to make their spatial choice on the basis of brightness discrimination or to learn always to choose the same side for attaining a goal. The present report describes these experiments and briefly considers their comparative behavioral implications.

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Methods

The experimental animals were juvenile specimens of *Panulirus argus* (Latreille) 8–12 cm. in body length. They were tested in an experimental discrimination box in which they were trained to walk forward (in air) to one of two compartments at the far end (Fig. 1). Each compartment had a large rectangular exit hole in its floor; one was open and led down to the aquarium below in which the lobster was normally kept; the other was closed by a wire screen which prevented

[1] Contribution number 290.

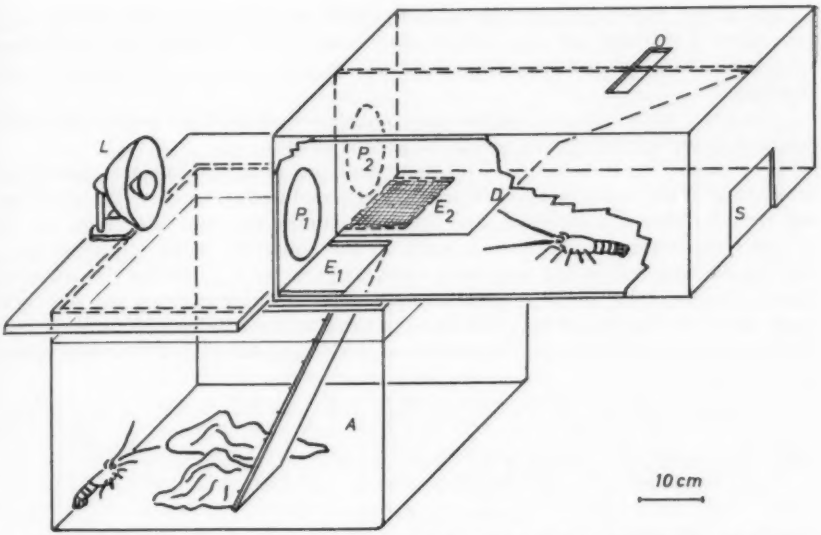

FIGURE 1. Two-choice spatial discrimination box used to train *Panulirus*. *A*, aquarium containing sea water; *D*, partition dividing front of box into two compartments; E_1, open exit from one compartment to aquarium below; E_2, blocked exit in other compartment; *L*, light source illuminating conditioning stimuli (P_1, P_2); *O*, slit for observing the lobster's behavior during tests; P_1, P_2, circular illuminated areas used in experiments training to brightness and those designed to train to plane of polarization; *S*, starting place for runs.

the animal's return home. The lobsters were observed through a peep hole and their course recorded on a floor plan of the box.

Two conditioning stimuli were presented when desired at a circular opening in the far wall of each compartment. These apertures were covered with translucent paper and illuminated from behind. The intensity of these stimuli could be altered by using additional layers of the translucent paper, and polarizers (Polaroid HN38 neutral polarizing filters) could be placed in the light paths to effect nearly 100% linear polarization of the illuminated areas.

Experiments were carried out as follows. The *Panulirus* were tested one at a time by placing them at the entrance of the experimental box facing the two goal compartments which were at the opposite end (Figs. 1 and 2). For animals which walked to the compartment with the open exit hole without entering the closed compartment a score of "correct" was kept; an error was recorded for those which walked into the closed compartment (score of "incorrect") and for those which stopped and remained anywhere in the back part of the box for five minutes ("nonspontaneous runs"), after which the lobsters were prodded into action and led in the correct direction with a small stick. When spontaneous locomotion was particularly circuitous or slow, this stick was also used to prod the lobster into action.

In addition to the error scores (in per cent per three trials) two other kinds of data were taken: (1) percentage of nonspontaneous runs and (2) percentage of runs in which tail-flexing reactions occurred.

In various experiments groups of individuals varying in number from 6 to 11 were given 2 to 6 tests per day, and the observations were repeated for 6 to 22 days. In certain cases the performances of trained and inexperienced individuals were compared.

The three main series of training experiments all followed the general procedure outlined above but differed in certain important details.

1. *Training to brightness.* Ten individual inexperienced lobsters which had never been in the experimental box before were used in these tests. The conditioning stimuli differed in intensity, and the more intense one was always on the side of the compartment with the exit open to the aquarium below (positive side). During the first 9 days I_{pos} was three times as great as I_{neg}; during the remaining 13 days I_{pos} was five times as great as I_{neg}. The first day each animal was tested three times, for the next 9 days five times per day, and for the last 12 days six times per day (about 119 runs per individual or 1188 altogether). To control against

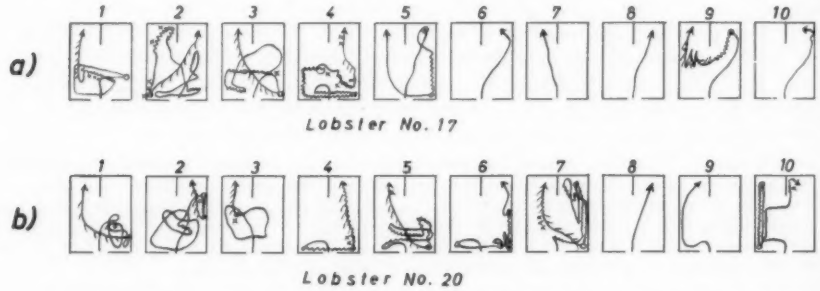

FIGURE 2. First ten runs of (a) a lobster (No. 17) which learned relatively quickly to discriminate brightness in choosing the right course to the open exit, and (b) a lobster (No. 20) which was slow in learning this task. The different parts of the animal's track are marked with the following symbol: full line = running forward; zig-zag line = running backward; small open circle = stop; cross hatching next to line = guiding by a stick; crosses = tail flexing movement.

the lobsters merely learning "side" the positive side was changed alternately from right to left for the first 12 days; then sides were shifted in random order for the next 10 days.

2. *Training to polarized light.* One series of measurements (A) was made on 11 inexperienced lobsters; a second series (B) was run with 10 experienced animals.[2] For Series A the box's wooden walls were unpainted. For Series B the walls of the experimental box were painted with aluminum paint in order to minimize reflection-refraction artifacts arising from differential effects of the walls on the total light pattern visible to the lobsters. The two conditioning stimuli had the same intensity and degree of linear polarization, but in one (the positive side) the e-vector was horizontal, while in the other it was vertical. Five tests per day per animal were made in each series. The horizontal polarization was regularly alternated from left (l) to right (r) in successive tests of Series B, but there were some irregularities in Series A which ran: rrlrlrlrlrrlrlrlrlrlrlrlrlr. Series A

[2] These had previously been used in the experiments on training to brightness.

lasted 6 days (about 30 tests per animal, total 327), Series B, 11 days (55 tests per animal, total 550).

3. *Training to side.* Six inexperienced lobsters were run in the experimental box for 9 days with the left compartment's exit open to the aquarium throughout. The intensities of the two light windows were equal and they were not polarized. Two tests were made per animal on the first day and three during the succeeding 8 days, making 26 per animal (total 156).

Results

A. *General behavior*

In the present experimental situation *Panulirus'* behavior was made up of activity sequences containing one or more of four locomotor components: (1) no locomotion, (2) forward walking, (3) backward walking, and (4) tail-flexing movements.[3] The actual paths followed were, of course, characteristically affected by the amount and rate of turning. Examples of the resulting trails are given as two series of 10 trials each for individual lobsters (Fig. 2).

Evidently these activity sequences differ in three major ways which are interrelated: (1) degree of spontaneity, (2) directness from starting point to end point, (3) particular locomotor components involved. Thus the most direct trails (Fig. 2a, Runs 7 and 8; Fig. 2b, Run 8) were started spontaneously, and consisted only of forward walking, at first straight ahead, then gradually turning towards the side with the open exit.

In slightly less direct paths the animal also started spontaneously and walked forward all the way but it turned more strongly towards the positive side, struck the side wall and followed this for a while until it turned in the opposite direction towards the exit (Fig. 2a, Runs 6 and 10; Fig. 2b, Run 9).

As the paths became less direct than this, more turning, stopping and backward walking appeared (Fig. 2a, Runs 5 and 9; Fig. 2b, Runs 4 and 10). Spontaneity was reduced and tail-flipping escape reflexes were evident. Finally, in the least direct runs the animals often did not leave the back part of the box spontaneously. In such cases they frequently had to be guided to the open exit with the stick. Backward walking, stopping and escape reactions occurred often (Fig. 2a, Runs 1, 2, 3 and 4; Fig. 2b, Runs 2, 5 and 7).

Some of the variation in these behavior patterns of *Panulirus* is due to individual peculiarities since the number and kind of certain loops, turns or backtracks tend to be similar in successive trials for the same lobster but to differ from one animal to another. The correlation of alterations in response with learning and the three types of training employed is considered in detail in the next section.

B. *Training experiments*

In running the two-choice maze of the present setup the short spontaneous paths over which the animals have walked directly forward to the open exit represent the best behavior if prompt return to sea water in their aquarium is considered the goal.

[3] These arise as strong flexions of the abdomen (Lochhead, 1961; Wiersma, 1961) which may result in the animal's projecting itself backward several centimeters in a response often termed an escape reflex.

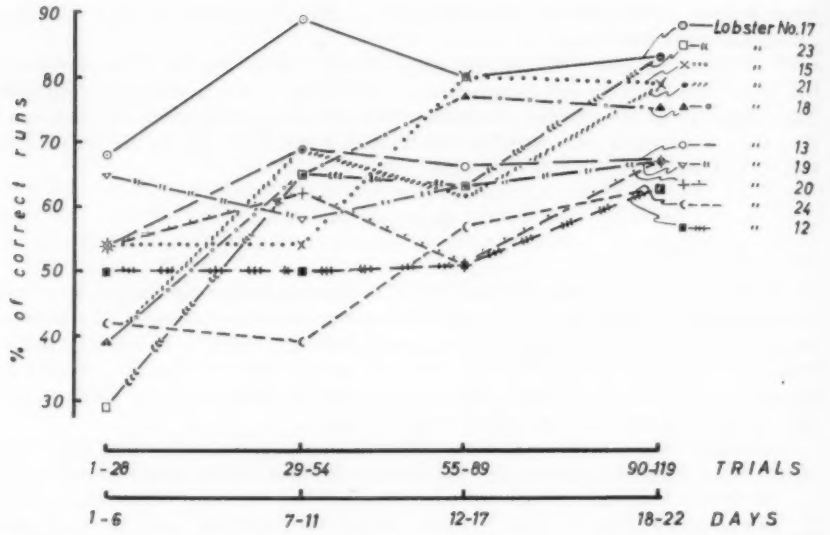

FIGURE 3. Learning curves for 10 individual lobsters training to brightness. Points indicate percentages of runs for the periods in trials (as well as days) specified on the abscissa.

Conversely, meandering runs with stops, walking backward, escape reactions and failure to reach the goal without guidance represent the worst performances. Under appropriate conditions of training and with test animals having a tendency to associate a conditioning stimulus with the goal, the poor responses shown initially by inexperienced animals improve progressively to a better performance level.

However, the amount of improvement and its rate during different parts of the training period were quite different in individual animals (Fig. 3). The factors involved in such variations remain largely unstudied. Nevertheless in certain experiments poor or insignificant improvement can be attributed to the development of certain preferences largely unaffected by the kind of associations tested. Thus in Series B of the training to polarized light, 5 of the 10 animals tested preferred one side of the box regardless of changes in the side of the open exit. This habit may be so rigid as to persist even when the positive side was never made the one preferred.

An example of this type of behavior, quite resistant to conditioning in the present setup, is shown in Figure 4. Here the alternation of positive side (a, 1; b, 1) from left to right was succeeded by the open exit being maintained only on the left (a, 2; b, 2). Despite this, Animal 12 continued to turn right, and even after a few correct responses, returned to its persistent incorrect right turn. Animal 15 showed a comparable predominance of right-hand turns for the greater part of the series but toward the end seemed to learn the left turn required to reach the positive side.

A summary of the numbers and types of runs made in each of the three kinds of training experiments is presented in Table I.

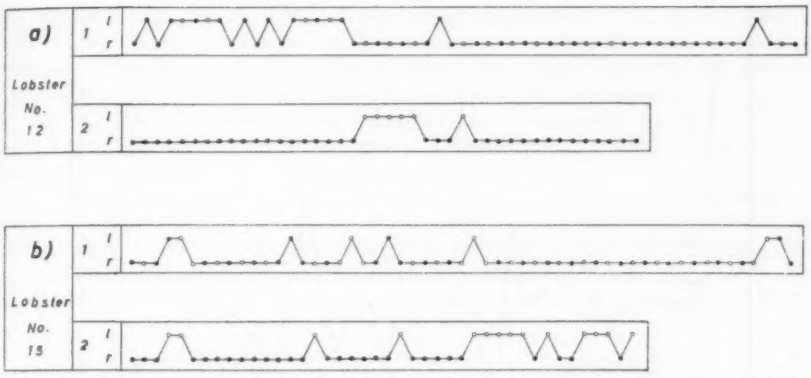

FIGURE 4. Serial representation of training runs of two experienced lobsters to polarized light (sequence from left to right). In series a1, b1 the open exit (positive side) was alternated regularly; in series a2, b2 the positive side was always to the left. Correct runs are represented by open circles, incorrect runs by filled circles. Development of a tendency to run towards the right side is shown by lobster No. 12 which kept this tendency even when the positive side remained left; lobster No. 15 did not, having finally changed to the positive side.

1. *Training to brightness.* Initially the average error score of the 10 animals tested was 93%. This had fallen to 35–40% after 30–40 trials and continued to diminish (but at a reduced rate) to about 20% by the end of the experiment (119 trials) (Fig. 5, error curve). The fraction of nonspontaneous runs was also high during the first trials (57%) but fell to about 10% after 10 trials and became zero for the last 40 trials (Fig. 5, curve of nonspontaneous runs). Similarly, the percentage of runs showing tail-flexing movement was maximum in the first trials (30%), declined irregularly during the next 30 trials and settled down to 0–7% in

TABLE I

Occurrence of tail-flexing movements during different kinds of runs in four series of training experiments

Runs	Training to brightness			Training to polarized light						Training to side		
				Series A			Series B					
	Total	With tail flexes		Total	With tail flexes		Total	With tail flexes		Total	With tail flexes	
		No.	%		No.	%		No.	%		No.	%
Correct	745	6	*1*	157	5	*3*	251	23	*9*	88	0	*0*
Incorrect*	394	82	*21*	156	66	*42*	271	86	*32*	33	5	*16*
Nonspontaneous	49	15	*31*	14	12	*86*	28	13	*46*	35	10	*29*
Total	1188	103	*9*	327	83	*26*	550	122	*22*	156	15	*10*

* Incorrect runs = error runs minus nonspontaneous runs.

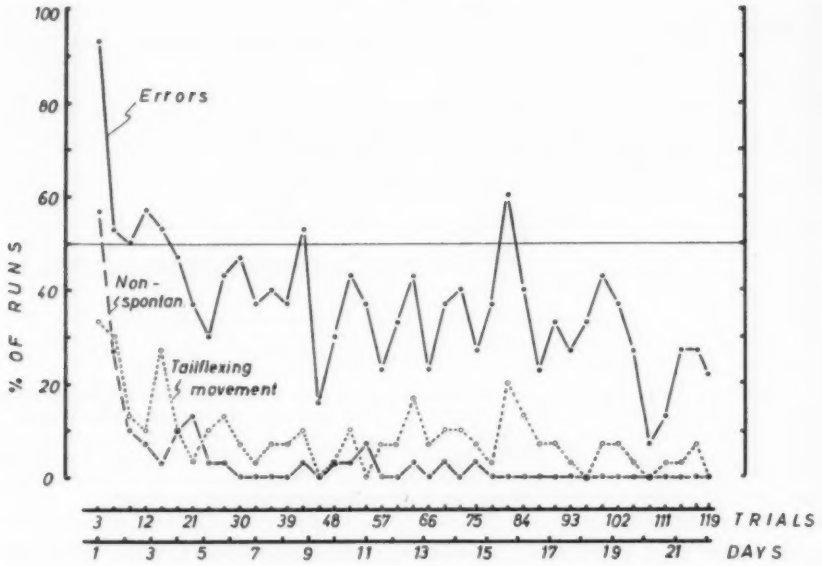

FIGURE 5. Mean learning curve for 10 lobsters training to brightness. Points represent percentages of the runs in three trials.

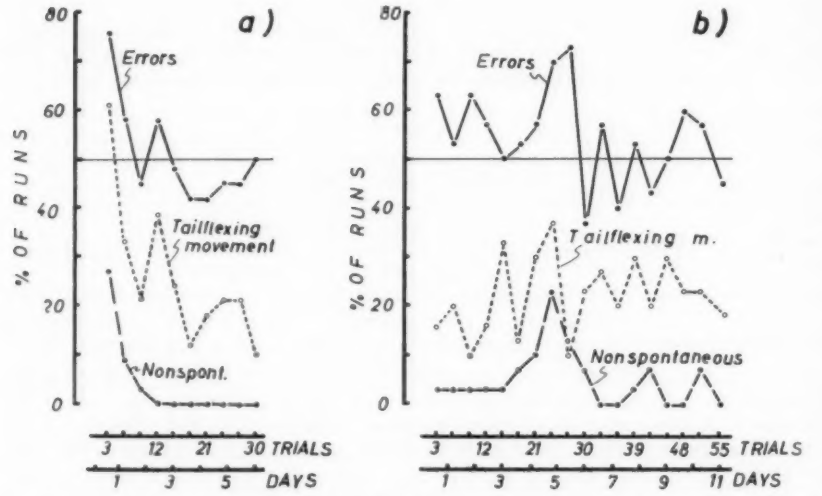

FIGURE 6. Mean learning curves for lobsters where training was attempted to plane of polarization. (a) Series A, 11 previously untrained animals, (b) Series B, 10 previously trained animals. Points represent percentages of the runs in three trials.

the last 30 trials (Fig. 5, curve of tail-flexing movement). The error curve and curve of tail-flexing movements at least show signs of flattening out, or even of a temporary upturn, starting with trial 61 when the regular right-left alternation of the positive side of the box was changed to a randomized sequence.

These results demonstrate that *Panulirus* can discriminate the light intensities used in the two compartments and utilize their position as a conditioning stimulus in goal seeking.

2. *Training to polarized light.* In both Series A (inexperienced lobsters) and Series B (experienced lobsters) the error scores were no better than random even at the end of the 6- and 11-day training periods (Fig. 6a, b). The inexperienced animals made a large number of errors (76%) in the first trials, but after 9 trials they made about 50% (Fig. 6a, error curve). Rapid decreases in the proportion

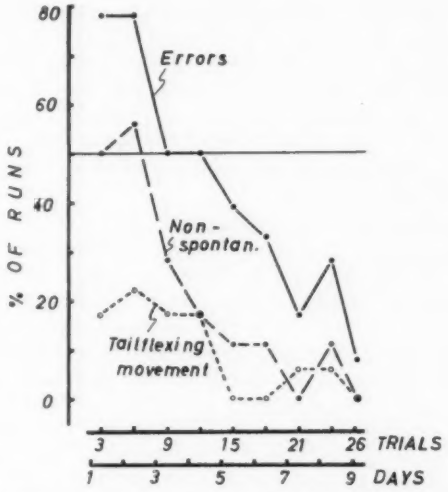

FIGURE 7. Mean learning curves for 6 lobsters training to side. Points indicate percentages of the runs in three trials.

of nonspontaneous runs and runs with tail-flexing movements also occurred in the inexperienced group (Fig. 6a, curve of nonspontaneous and tail-flexing movement runs).

In contrast, with the previously trained animals all three of these scores were about at the same level in the first as in the last trials (Fig. 6b). Although these two series showed that spontaneity and tail-flexing movements reached levels comparable to those attained in the brightness training (Fig. 5), they give no indication that the two tested orientations of the *e*-vector of polarized light could be used as a conditioning stimulus to discriminate the spatial choice required.

3. *Training to side.* The general behavior of test animals in the above experiments suggested that they readily could learn the side with a reward since they often went toward that compartment which had been the positive one in the previous test.

This was confirmed by the results of experiments carried out to study this type of training. The error score was over 78% at first but decreased precipitously and quite steadily thereafter. It was 50% after 9 trials and about 10% after 26 trials at the end (Fig. 7, error curve). A similar course was run by the proportion of nonspontaneous runs which started at 50% and decreased after 15 trials to 11%. The fraction of runs with tail-flexing movements showed a general trend to decrease from about 20% initially to zero in the last trials.

Discussion

Experience indicates that rate and extent of learning in various animals depend strongly on the particular problem tested and the sensory modalities involved in the afferent components of the process. Also the strength and type of drive and reinforcement involved obviously will markedly affect learning (Hull, 1952). Nevertheless, it seems clear in the present experimental situation that *Panulirus* did not learn even at best to make two-choice spatial discrimination either very quickly or very accurately. Mean error scores for a day never were substantially less than 20% and even these took more than 20 trials to achieve. Thus in the training-to-side experiment the error score dropped markedly below 20% only after 26 trials (Fig. 7) and in the training-to-brightness series an error score markedly below 20% first appeared after 108 trials (Fig. 5).

The differences in learning shown in these two sets of training experiments may reflect the relative importance of different sensory modalities for the type of conditioning involved. The more rapid achievement of $< 20\%$ errors in choosing one side than in discriminating between the two circular areas of different brightness suggests that some tactile or kinesthetic cues are more important than visual ones in finding the open exit hole. Such an interpretation is supported by the cases cited above (Fig. 4) where persistent preference for one side was manifest in certain individual *Panulirus* and by previous work on the fiddler crab *Uca* (Schwartz and Safir, 1915) and the crayfish *Pacifastacus* (Gilhousen, 1927) which demonstrated turning predominantly in one direction or following of one wall. Such a preponderant influence of mechanoreceptor afferent information for *Panulirus* is consonant with the animal's normal rocky surroundings in submarine holes and crevices, coupled with its essentially nocturnal habit.

The lack of evidence for the lobsters learning to discriminate between vertically and horizontally polarized light sources is consonant with the conclusion that visual cues are not as significant as certain others in the present spatial choice problem. However, it does not seem to reflect the finding in the crab, *Ocypode,* that vertically polarized light has a significantly greater effect on eyestalk displacement than horizontal polarized light of the same photometric intensity (Schöne and Schöne, 1961).

Previous attempts with other animals to demonstrate discrimination of different planes of polarization by training techniques have yielded both positive and negative results. In the pigeon, food-training discrimination tests gave no evidence for polarized light perception but indicated good light intensity discrimination (Montgomery and Heinemann, 1952), thus resembling some of the present results with *Panulirus*. On the other hand, in the honeybee a significant ability to discriminate vertically from horizontally polarized light was reported on the basis of food-seeking

training (Stockhammer, 1956).[4] Reward and punishment training in *Octopus* has similarly demonstrated that animal's ability to discriminate vertical from horizontal polarized light as well as between oblique planes of polarization at 45° and 135° to the vertical (Moody and Parriss, 1960, 1961).

However, the present failure to get discrimination learning to polarized light cannot be taken as evidence that the animal does not perceive the plane of polarization, as a great variety of arthropods, including decapod crustaceans, must do (Stockhammer, 1959; von Frisch, Lindauer and Daumer, 1960; Jander and Waterman, 1960; Waterman, Jander and Daumer, unpublished; Schöne, unpublished data). Negative evidence of the sort obtained here merely demonstrates an absence of training effect in the current experimental situation without decisive prejudice to the more general question.

In the learning curves for the error scores of inexperienced lobsters (Figs. 5, 6a, and 7), the occurrence of initial levels significantly above the random choice value of 50% results directly from the way errors were counted. The inclusion of nonspontaneous runs as errors contributed substantially to the initial excess over what would be expected, since inexperienced animals frequently required prodding to initiate and maintain walking.

On the other hand with experienced animals the initial error scores were near 50% (Fig. 6b) and the nonspontaneous runs were much less frequent during the first trials. This raises two points of interest. (1) Retention of training must occur for the period between experiments (3 days). (2) Learning, as evidenced by the reduction in nonspontaneous runs, took place even when the *Panulirus* were not learning to find the open exit. Such data suggest that the test situation consists of at least two stages: (1) learning to walk forward (probably related to the general factor of drive or appetitive behavior (Tinbergen, 1951), and (2) learning to discriminate the positive compartment.

Another point of interest is the correlation of tail-flexing movements with inexperience and with progress in learning. The tail-flexing movements appeared to be characteristic of inexperienced individuals (Figure 2 and the first trials of the appropriate curves of Figures 5 and 7) and of those showing little tendency to learn (Fig. 6). In both of these cases tail-flexing is correlated with error runs. The detailed correlations of tail-flexing movements with correct, incorrect and nonspontaneous runs are shown in Table I which indicates that tail-flexing movements were most prominent in nonspontaneous runs, less so in incorrect runs, and at a minimum in positive runs where the open exit was reached spontaneously.

The protocols show that tail-flexing movements were particularly associated with "indecisive" behavior. Thus, if the lobster (1) was inactive unless prodded with the stick, (2) chose the negative compartment and found the exit blocked, (3) made turns in more than one direction, stopped or walked backwards, then tail-flexing movements were likely to appear suddenly. In cases (1) or (2) above the tail-flexion response could be interpreted as an escape reaction from a threatening situation as it often appears to be for lobsters in the sea when strong external stimulation occurs (such as the nearby appearance of a large fish). However, when the same reaction appeared as a component of "indecisive" behavior patterns lacking

[4] In these experiments no control is mentioned against the possibility that intensity artifacts due to differential reflection of the polarized light were involved in the training process.

any obvious external threat, the tail-flexing movement would seem to be a displacement activity in the ethological sense (Eibl-Eibesfeldt and Kramer, 1958; Schöne, 1961). Thus, its diminution with experience could be considered as habituation to initially startling or conflicting situations.

Summary

1. Juvenile spiny lobsters (*Panulirus argus*) could be trained in air in a two-choice discrimination box to use two stimulating lights which differed in intensity by a factor of 3–5 times in learning to reach an open exit hole leading to an aquarium containing sea water.

2. Comparable training procedures yielded no better than chance selection of the two compartments (one with the open exit, the other with its exit blocked) when the conditioning lights were of equal intensity but were both linearly polarized, one horizontally, the other vertically. Demonstration that lobsters did not use polarization pattern in this spatial discrimination situation does not prove that the plane of polarization was not perceived by *Panulirus*.

3. Learning was more rapid than it was to intensity differences when the animals were trained always to go to one side of the discrimination box to reach the open exit. For intensity discrimination initial high percentage errors were reduced to less than 20% only on the twentieth day after 104 trials per animal, whereas for training to side less than 20% errors were achieved after 20 trials per animal.

4. Learning indicated by the curve of mean error scores per day resembles an exponential decay function and is accompanied by decreases in percentage of nonspontaneous runs and runs showing tail-flexing movements. Tail-flexing movements were correlated positively with error runs and occurred as a result of "indecisive" behavior.

5. Learning indicated by reduction in percentages of nonspontaneous runs showed retention of training between experiments and continued in cases where exit discrimination was not being learned. Hence over-all training involved: (a) learning to walk directly forward, and (b) learning to discriminate the side with the open exit.

LITERATURE CITED

Bethe, A., 1898. Das Centralnervensystem von *Carcinus maenas*. II. Teil. *Archiv. mikroskop. Anat. u. Entwicklungsges.*, **51**: 382–452.

Bush, R. R., and W. K. Estes (Eds.), 1959. Studies in Mathematical Learning Theory. 432 pp., Stanford University Press, Stanford, California.

Datta, L-E., S. Milstein and M. E. Bitterman, 1960. Habit reversal in the crab. *J. Comp. Physiol. Psychol.*, **53**: 275–278.

Eibl-Eibesfeldt, I., and S. Kramer, 1958. Ethology, the comparative study of animal behavior. *Quart. Rev. Biol.*, **33**: 181–211.

von Frisch, K., M. Lindauer and K. Daumer, 1960. Über die Wahrnehmung polarisierten Lichtes durch das Bienenauge. *Experientia*, **16**: 289–301.

Gilhousen, H. C., 1927. The use of vision and of the antennae in the learning of crayfish. *Univ. Calif. (Berkeley) Publs. Physiol.*, **7**: 73–89.

Hilgard, E. R., 1956. Theories of Learning. 2nd Edition, 563 pp., Appleton Century Crofts Inc., New York.

Hull, C. L., 1952. A Behavior System. 372 pp., Yale University Press, New Haven, Connecticut.

JANDER, R., AND T. H. WATERMAN, 1960. Sensory discrimination between polarized light and light intensity patterns by arthropods. *J. Cell. Comp. Physiol.*, **56**: 137–159.

LOCHHEAD, J. H., 1961. Locomotion. *In:* The Physiology of Crustacea (edited by T. H. Waterman). Vol. II, pp. 313–364, Academic Press, New York.

MONTGOMERY, K. C., AND E. G. HEINEMANN, 1952. Concerning the ability of homing pigeons to discriminate patterns of polarized light. *Science*, **116**: 454–456.

MOODY, M. F., AND J. R. PARRISS, 1960. Discrimination of polarized light by *Octopus*. *Nature*, **186**: 839–840.

MOODY, M. F., AND J. R. PARRISS, 1961. The discrimination of polarized light by *Octopus:* a behavioural and morphological study. *Zeitschr. vergl. Physiol.*, **44**: 268–291.

SCHÖNE, H., 1961. Complex behavior. *In:* The Physiology of Crustacea (edited by T. H. Waterman). Vol. II, pp. 465–520, Academic Press, New York.

SCHÖNE, H., AND H. SCHÖNE, 1961. Eyestalk movements induced by polarized light in the ghost crab, *Ocypode*. *Science* (in press).

SCHWARTZ, B., AND S. R. SAFIR, 1915. Habit formation in the fiddler crab. *J. Animal Behavior*, **5**: 226–239.

STOCKHAMMER, K., 1956. Zur Wahrnehmung der Schwingungsrichtung linear polarisierten Lichtes bei Insekten. *Zeitschr. vergl. Physiol.*, **38**: 30–83.

STOCKHAMMER, K., 1959. Die Orientierung nach der Schwingungsrichtung linear polarisierten Lichtes und ihre sinnesphysiologischen Grundlagen. *Ergeb. Biol.*, **21**: 23–56.

THORPE, W. H., 1956. Learning and Instinct in Animals. 493 pp., Methuen, London.

TINBERGEN, N., 1951. The Study of Instinct. 228 pp., Oxford University Press, London and New York.

WARDEN, C. J., T. N. JENKINS AND L. H. WARNER, 1940. Comparative Psychology. Vol. II. 1070 pp., Ronald Press Company, New York.

WIERSMA, C. A. G., 1961. Reflexes and the central nervous system. *In:* The Physiology of Crustacea (edited by T. H. Waterman). Vol. II, pp. 241–279, Academic Press, New York.

ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1961

ABSTRACTS OF SEMINAR PAPERS

JUNE 26, 1961

Varieties of ephaptic transmission in crayfish. HARRY GRUNDFEST AND AKIRA WATANABE.

The septal membranes of the abutting segments of the lateral giant axons of the crayfish nerve cord form bidirectional ephaptic junctions. The properties of the junctional membranes are described accurately in terms of a simple (non-reactive) resistance which links the reactive, electrically excitable and spike generating membranes of the two segments. Thus, the septal membranes show no rectification, at least for a range of polarization of ±25 mv., nor do they respond with electrogenesis to depolarization by 70 mv. The electrical inexcitability of these ephaptic membranes accounts for the bidirectional transmission of the junctions. In contrast, the membranes of the unidirectional ephaptic junctions between the cord and root giant axons have an electrically excitable component which is responsible for the high degree of rectification of these membranes. The available data are insufficient to indicate whether the conductance change is due to K-activation, as in frog slow muscle fibers (Belton and Grundfest, this issue) or to Cl-activation, as in *Raia* electroplaques. Bidirectional ephaptic junctions are also formed by the commissural connections between the branches of corresponding segments of the two lateral giant axons. Since these connections form a ladder of cross-linkages at the different segmental levels, circus propagation of impulses from one axon to the other is possible. These ephaptic pathways can give rise to reverberating activity of both lateral giant axons at rates as high as 400 impulses per second. This activity presumably can initiate corresponding activity in other neurons of the nerve cord.

JULY 3, 1961

The advance of electrical models for cells and axons. KENNETH S. COLE.

The electrical models of cells and axons have advanced rather steadily from the model for the red blood cell given by Fricke in 1925, which demonstrated the molecular dimensions of living membranes, to the detailed model for the squid giant axon given by Hodgkin and Huxley in 1952 in which much of classical electrophysiology is explained in terms of membrane ionic conductances obtained from voltage clamp experiments.

JULY 10, 1961

The components of the resting potential in crayfish and lobster muscle fibers. LUCIEN GIRARDIER, JOHN P. REUBEN AND HARRY GRUNDFEST.

In short range experiments involving changes of external potassium, lobster muscle fibers depolarize with a slope of 58 mv./decade increase of K, according to the thermodynamic (Nernst) relation. Under similar experimental conditions, crayfish muscle fibers do not manifest the theoretical $E_m - \log K$ relation. The latter is attained, however, when thermodynamic conditions are established, *e.g.* by keeping the product $K \times Cl$ constant. Furthermore,

the crayfish fibers exhibit a long-lasting electrochemical disequilibrium after modification of the bathing solution. Thus, remarkably slow transient potential changes are induced by altering the Cl-content of the medium. These properties are consonant with the high membrane resistance of crayfish muscle fibers and with their high volume-to-surface ratio, and the preparation is thus particularly suitable for the study of the pharmacological effects on the ionic fluxes which can be deduced from the kinetics of the membrane potential. The synaptic agents, GABA and picrotoxin, which respectively initiate and block activity of the inhibitory synaptic membrane in these fibers, respectively increase and decrease the membrane resistance and the net Cl-flux in the resting membrane. Although the inhibitory synapses of the fibers operate through increased Cl-conductance, the effects of the drugs on net Cl-flux are due to complementary actions on the non-synaptic membrane component. They are produced only by high concentrations of the agents, well after the maximal effects on the synaptic membrane had been attained. Barium, which converts the normally graded responses of the fibers to spikes and also increases the membrane resistance, decreases the transport number for K. Procaine, which also converts the graded responses to spikes, but has little or no effect on the resting resistance, exerts no clear-cut action on any one ion flux. In lobster muscle fibers, by comparison, the synaptic drugs and Ba have similar actions on the membrane resistance. However, only Ba modifies the E_m – log K relation of lobster muscle fibers, as may be expected, since the relation in these muscle fibers is determined primarily by the K.

JULY 11, 1961

Temperature adaptation in Drosophila melanogaster pupae. ROGER MILKMAN.

Day-old (at 23° C.) *D. melanogaster* pupae respond to treatment at 39.5°–41.5° by showing quantitative defects in the posterior crossvein. Longer treatments lead to death. Both responses have a Q_1 of 2.3, and both depend upon the temperature at which the first day of pupal life is spent.

The higher the temperature, the greater the resistance, both to crossvein defect production and to death. Temperatures at 1° intervals between 20° and 30° have been tested. The *rate of increase* in resistance per degree rises greatly with higher temperatures in the range.

The temperature coefficient measurements, previously published in detail, imply that these crossvein defects result from thermal protein denaturation. Moreover, duration-response curves for crossvein defects at 40.5°, using pupae aged at 23° and at 28°, are now shown to be parallel, the duration threshold being greater for the 28° pupae. This implies similar protein denaturation rates, and that the 28° pupae had more of the relevant protein to begin with.

Since resistance to morphological effects and death is similarly temperature-dependent at various pupal stages in *D. melanogaster* and other *Drosophila* species, it is suggested that one mechanism of temperature adaptation may be the change in concentration of certain proteins, or perhaps proteins in general.

Short prior exposures (5–60 minutes) to 32°–38° obliterate the crossvein response and defer death. Production of crossvein defects and both forms of temperature adaptation have been shown in *D. virilis, D. willistoni, D. pseudoobscura,* and *D. busckii.*

This work supported by grants from NSF and NIH.

Endoplasmic reticulum in the asters of invertebrate eggs. LIONEL I. REBHUN.

Electron microscopic evidence was presented showing an orientation of the endoplasmic reticulum in the asters of the egg of *Spisula solidissima.* Such an orientation can be seen during formation of asters of the first polar body spindle after germinal vesicle breakdown and during aster formation, just prior to first cleavage. In eggs which have been centrifugally stratified during periods in which asters are present, the endoplasmic reticulum can be seen as a layer 4 to 5 microns wide, tightly pressed beneath the lipid layer. This corresponds to a layer distinguishable in the stratified egg with the light microscope and in the same sublipid position. During redistribution after stratification, asters reappear usually in the form of "one-sided" radiate bodies at first, and later, as full asters. In the electron microscope the endoplasmic reticulum shows a similar one-sided radial orientation, later changing to a

fully radiate body, again suggesting that an important component of the aster is the endoplasmic reticulum.

The orientation of centrioles in dividing cells and its significance. DONALD P. COSTELLO.

The centrioles of the resting first cleavage metaphase of the eggs of the acoel, *Polychoerus carmelensis*, are curved rods about 5 microns long, oriented at right angles to each other and to the spindle axis (Costello, 1961). By light microscopy a smaller rod, projecting at right angles from the middle of each centriole, but at an oblique angle to the spindle axis, can be discerned. At the end of this accessory rod is a small, clear vacuole, containing a stainable (with iron haematoxylin) granule. Extending into the centrosomal area, away from this vacuole, are other similar vacuoles, each with a granule. As Heidenhain (1894) pointed out, and Schreiner and Schreiner (1905) demonstrated, centrioles in many species reproduce by budding, with the daughter centriole produced at right angles to the mother centriole. The small rods are therefore centriolar buds (daughter centrioles) which will grow and separate, presumably at the angle indicated by their mode of origin, from the mother centriole.

Comparing this centriolar orientation and behavior (including daughter centriole origin) in *Polychoerus* with that in the spermatocyte divisions of Gryllidae (Johnson, 1931) and Hemiptera (Payne, 1927) in relation to the arrangement of daughter cells, leads to a new hypothesis, which may account, in part, for cleavage patterns:

(a) The orientation of the centrioles and their mode of reproduction determines the path of separation of daughter centrioles from mother centrioles (or of daughter centrioles from each other).

(b) This, in turn, determines the position of the main axis of the spindle for the next division, and hence, the relative position of the daughter cells with respect to each other.

(c) This arrangement of daughter cells is maintained, for a time, by the primary cell connective, of which the spindle remnant is the significant portion.

(d) These relations obtain in the absence of secondary intervening factors.

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JULY 18, 1961

The nature of lamprey eel fibrinopeptide material. RUSSELL F. DOOLITTLE.

When various components of the mammalian blood coagulation scheme are reacted with components from lower vertebrates, the ensuing clotting times are invariably longer than when all the components come from the same or closely related animals. An investigation of the chemical nature of this "species" specificity was undertaken, using purified preparations of lamprey eel thrombin and fibrinogen and their mammalian counterparts. Human thrombin will completely clot lamprey eel fibrinogen, but the reaction proceeds at only 50% of the rate of human thrombin clotting bovine fibrinogen. Lamprey eel thrombin will clot bovine fibrinogen at less than 10% the rate at which it clots lamprey fibrinogen.

The clotting of mammalian fibrinogen by mammalian thrombin is effected by the enzymatic release of two different negatively charged peptides. It has been postulated that the negative charge clusters keep the fibrinogen molecules mutually repelled and that their loss permits aggregation of the monomeric units. There has been some dispute as to whether or not it is necessary to cleave both peptides in order for the fibrinogen to completely clot. When lamprey eel thrombin clots bovine fibrinogen, only peptide A is released, in spite of the fact that over 90% of the original protein is incorporated into the clot. Human thrombin and lamprey thrombin yielded identical products when exposed to lamprey fibrinogen. One electronegative peptide and one peptide bearing a positive charge at neutral pH were detected. The amino acid composition of the two lamprey peptides was determined and compared with bovine fibrinopeptides. The nature of restricted and permissive mutations in the evolution of the thrombin-fibrinogen interaction was discussed. The details and methods of this work will be published elsewhere.

Equal and unequal effects of unit dosages of ultraviolet and x-ray irradiation of Arbacia gametes as recorded by cinephotomicrography. RALPH HOLT CHENEY AND CARL CASKEY SPEIDEL.

Eggs and sperm were subjected separately to either 2537 Å ultraviolet or x-ray irradiation. UV-rayed gametes were exposed for periods ranging from one-half second to 4 minutes, the rate of delivery being 103 ergs per sq. mm. per second. Gametes exposed to x-rays received from 4 to 120 kr. Fertilization combinations were then made and ensuing developmental stages recorded by cinephotomicrography as follows: (1) elevation of fertilization membrane, (2) first cleavage, (3) 5-hour cleavage, (4) initiation of blastular motion at about 8 hours, (5) 24-hour development. Motion picture scenes of stages 3, 4, and 5 were especially useful in demonstrating that there was a striking difference in the relative effects induced by UV treatments as compared with x-ray treatments.

A unit UV dose to eggs caused *far less* developmental delay and injury than an equal UV dose to sperm. Even a 4-unit dose to eggs caused less damage than a 1-unit dose to sperm. An 8-unit dose to eggs, however, induced delay and damage approximately equivalent to a 1-unit dose to sperm. With x-ray irradiation, on the other hand, although a unit dose to eggs was followed usually by less damage than an equal dose to sperm, the difference was much less marked. A 2-unit dose to eggs, or even less, was sufficient to induce delay and damage equivalent to a 1-unit dose to sperm. *Thus, there was a sharp contrast between the two types of irradiation.* A ratio of 8:1 describes the relation between the dosages of UV irradiation to eggs and sperm, respectively, to induce equivalent effects; a ratio of not more than 2:1 describes this relationship for x-ray.

Time-lapse motion pictures of stages 1 and 2 illustrate varieties of fertilization membrane elevation (concentric, eccentric, and partial), also movements of intracellular structures.

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JULY 25, 1961

Fine structure of an unusual sperm in the flatworm Plagiostomum. A. KENT CHRISTENSEN. *(See Lalor Fellowship Reports).*

Observations on the Golgi apparatus and related lysosomes. ALEX B. NOVIKOFF.

Nucleosidediphosphatase has been localized in Golgi lamellae and a staining method developed that permits delineation of the Golgi apparatus from sites of high acid phosphatase activity. In epididymis and many other cells the acid phosphatase-rich lysosomes show a close topographical relation to the Golgi lamellae. Essner's observations in our laboratory on differentiated hepatoma cells suggest that there may be a "membrane flow" to and from the Golgi apparatus: elements of the endoplasmic reticulum (ER) transform into Golgi cisternae (in one case, this apparently involves flattening of individual smooth-surfaced sacs derived from the rough ER; in the other, Golgi cisternae appear to arise *in situ* by transformation and reorientation of the smooth ER); the cisternae enlarge and separate into vesicles (lysosomes) containing condensed secretory products. Light microscopy and electron microscopy show that the ER (and nuclear membrane) of these cells, and another interesting group, possess nucleosidediphosphatase activity, and that the acid phosphatase reaction product is localized in the lysosomes of these tumor cells. The lysosomal hydrolases may be involved in the biochemistry of secretory granule condensation. How the enlarged Golgi vesicles acquire hydrolytic enzymes is unkown, but there is some suggestion that this may occur via small lysosomes (pinocytosis vacuoles?). In the zymogenic cells of parotid, pancreas and jejunum, acid phosphatase-rich granules are restricted to the Golgi area, and thus only the newly formed secretory granules appear to be lysosomes. Of many cells tested, only the spermatogonia and their derivatives always show high acid phosphatase activity in the Golgi apparatus. Acid phosphatase is present in the acrosome (frog sperm) which may be considered a specialized lysosome with a decisive role in fertilization.

Functional analysis of the corpus allatum of the insect, Leucophaea maderae, with the electron microscope. BERTA SCHARRER.

In adult females of *Leucophaea*, periodic changes in corpus allatum activity are paralleled by structural variations whose details become particularly evident in electron micrographs. In the inactive gland, which due to inhibitory action of the brain does not furnish gonadotropin, the nuclei are densely packed and surrounded by scarce cytoplasm; their chromatin appears clumped. Neighboring cells interdigitate by elaborate foldings of the cell boundaries. Furthermore, the cells interlock by means of long, gradually thinning processes. Following cessation of inhibition, intermediate stages lead to the fully activated corpus allatum and the concomitant maturation of eggs. A considerable increase in cytoplasmic content of the corpus allatum cells is accompanied by gradual straightening of their cell membranes. The number of mitochondria rises, and endoplasmic reticulum appears in the form of juxtanuclear spherical units which may be paired. In addition, small clusters of unattached ribosomes become more numerous, while Golgi elements remain inconspicuous. Electron-dense cytoplasmic granules are especially noticeable at the surface of the gland. With increasing nuclear diameter the chromatin becomes more evenly distributed. One, or not rarely two large nucleoli show a characteristic morphology. The folding and straightening of the cell membrane, as its content shrinks and expands with each reproductive cycle, demonstrates its structural identity. The stellate shape of the corpus allatum cells, which is observed also in fresh tissue dissociated by means of trypsin, seems to be maintained throughout the changes of volume. Branches of the basement membrane which ensheaths the corpus allatum and the corpus cardiacum penetrate between the corpus allatum cells. Some of these processes enclose neurosecretory elements. This morphological intimacy between the two organs reflects their close functional relationship.

JULY 31, 1961

Biological transducer mechanism. WERNER R. LOEWENSTEIN.

The data available presently on charge transfer through the receptor membrane of the nerve ending of Pacinian corpuscles can be fitted to the following excitation scheme: the receptor membrane separates two media of different ionic concentration. Mechanical stimulation causes the conductance of the membrane to increase in a relatively non-selective manner, and ions to flow through along their electro-chemical gradients. An activation energy barrier of about 16,000 cal./mole limits this process.

The membrane reaction that leads to the conductance change is highly localized. It is localized at and confined to the membrane spot that has been stimulated mechanically, and does not spread to adjacent spots within the same receptor membrane by local circuit excitation. This stands in striking contrast to the well-known propagated nature of excitation of the adjacent axon membrane that gives rise to the action potential. The peculiarity of the receptor membrane is that excitation is produced by the mechanical stimulus alone, but not by the resulting local flow of current. As a result, the amount of charge transferred through the membrane, namely the generator current, increases with area of membrane excited. The area-generator current relationship is non-linear; the current increases progressively at a diminishing rate with excited area. The area of membrane excited (that is, the lumped membrane conductance) is the main factor, if not the only one, in determining the intensity of the generator current.

Repeated excitation leads to a transient inactivation of the receptor membrane. The effect manifests itself as a reversible depression in generator current which has low energy requirements and is independent of resting membrane potential. Like excitation, inactivation is a localized process.

AUGUST 7, 1961

Current voltage relations in Nitella. UICHIRO KISHIMOTO.

The measuring cell and the voltage clamp setup (Cole and Moore, 1960) for squid giant axon was used for the *Nitella* experiment, because the size and shape of *Nitella* internode are just

about same as those of squid axon. Two microtips were inserted into vacuole of *Nitella* internode instead of the axial wire-microtip system used in the squid giant axon, since penetration of the 50- to 100-μ axial wire caused a sudden rapid loss of vacuolar sap due to high osmotic concentration gradient between inside and outside of *Nitella*. One microtip is used for monitoring the potential between vacuole and the reference electrode just outside of *Nitella* membrane. Another is used for current supplying through the membrane. Space clamp condition is achieved with this arrangement, since the output of the feedback amplifier appears to be a perfect square wave, which is probably due to a large space constant of *Nitella* (2–3 cm.). The membrane current during step potential change is very similar to that of squid giant axon. The membrane current consists of three components. The first one is the sudden rise or fall of current which corresponds to leakage current, the second is the transient inward current and the third is the steady outward current which increases slowly with time and finally approaches to a constant level. Ions which contribute to the leakage current are not identified as yet. However, there is a good reason to suppose that the second one corresponds to the marked increase of chloride efflux and the third one to that of potassium efflux.

Increase of outside chloride concentration to 10 m*M* causes a reduction of the transient inward current to about half. Increase of outside calcium concentration to 10 m*M*, maintaining the outside chloride concentration constant, brings about a longer duration for action potential without changing the height of the latter. The transient inward current is not influenced by high calcium. Threshold for excitation was increased and the leakage current decreased markedly. Decreasing the temperature to 7° C. promptly increased the leakage of the *Nitella* membrane and the threshold for excitation was increased greatly. The recovery from the effect of lowered temperature takes about an hour. Application of prepulse (subthreshold intensity) of 1–3 seconds in duration has no influence on the inward and outward currents, but the leakage current tends to decrease for hyperpolarization prepulse and to increase for depolarization prepulse.

August 8, 1961

The physiological basis of acclimation of Tetrahymena to high NaCl medium. Philip B. Dunham.

It has been known for some time that fresh-water ciliates can acclimate to concentrated media. However, little was known of the physiological basis of acclimation. *Tetrahymena pyriformis* strain W was acclimated to a 1%NaCl–2% proteose-peptone medium, and compared to a normal (unacclimated) culture of the same animals. An investigation of ion regulation in normal *Tetrahymena* has been reported in this journal, in which it was shown that these animals have a sodium extrusion mechanism with a saturation level at 20 m*M*. medium Na and an apparent free sodium space in the cells of 48%. The acclimated culture was started without intermediate steps. Only 5% of the normal animals survive to divide in the high NaCl medium. The acclimated animals have a much lower sodium content (39 m*M*./l. of cells) than do normal animals equilibrated in high NaCl medium (103 m*M*./l. of cells). The potassium and chloride contents of the two cultures of animals are not significantly different. A comparison of the relationships between cell sodium and medium sodium in acclimated and normal animals suggested two kinds of mechanisms operative in the acclimation: (1) a decrease in apparent free sodium space in the cells from 48% to 20%; (2) an increased saturation level of the sodium extrusion mechanism from 20 m*M*. Na to 45 m*M*. The acclimated animals have a slower growth rate, are smaller, and are differently shaped.

Excretion rate of radio-isotopes as indices of metabolic rates in nature: biological half-life of zinc-65 in relation to temperature, food consumption, growth and reproduction in arthropods. Eugene P. Odum.

Laboratory experiments and a preliminary field experiment with two species of terrestrial insects (*Tenebrio molitor* and *Oncopeltus fasciatus*) and a marine isopod (*Idothea baltica*) indicate that the excretion rate of tracer amounts of Zn^{65} is proportional to certain activity rates, especially food consumption and egg production rates. Individuals were allowed to

ingest contaminated food or water in a "tagging chamber," then transferred to a clean environment where the loss of radioactivity was followed by making periodic whole body counts in a well scintillation detector. Semi-log plots of radioactivity against time strongly indicated two pools of isotope: (1) a non-assimilated pool which is lost rapidly at a rate dependent on the level of the original "tag" and (in aquatic environments) on the physio-chemical nature of the environment, and (2) an assimilated pool which is distributed throughout the tissues of the body with some concentration in digestive organs and gonads and which is excreted much more slowly at a rate more directly related to metabolic processes. Biological half-life ($Tb_{1/2}$) of the assimilated pool varied from as long as 250 days in inactive adults kept at low temperatures to 20 days in active egg-laying *Oncopeltus* females or rapidly growing *Tenebrio* larvae. Young adult *Tenebrio* kept for 30 days at 10°, 20°, and 30° C. averaged 163, 40, 23 days $Tb_{1/2}$, respectively, which is roughly inversely proportional to expected metabolic rate at these temperatures. $Tb_{1/2}$ of 5 adult *Oncopeltus* which had been confined to small bottles in the laboratory decreased 5-fold (125-25 days) when released for 10 days in an outdoor habitat, indicating much greater activity in the field. It is concluded that the rate of loss from the assimilated pool provides a "metabolic clock" for comparing laboratory and field rates of energy flow in arthropod populations.

Supported by AEC contract AT (07-2)-10 and NIH 2G-535 (MBL Ecology Training Program).

August 22, 1961

In vitro culture of human tumors on explants of chick embryo organs. Emilienne Wolff and Etienne Wolff.

Previous researches of our laboratory have shown that it is possible to culture malignant cells from mammals on chick embryonic organs explanted *in vitro*. Mesonephros is one of the most favorable organs for tumor proliferation. Up to now, cells originated from fresh mouse tumors and from standard human strains have been cultured by this method.

Recent improvements of our technique made it possible to obtain new developments of this investigation.

1. A thin membrane, the vitelline membrane of the hen's egg yolk, is placed between mesonephric explants and cancer cells, originated from the human strains HeLa, KB, Fogh, J III, Detroit 6 and Detroit 116. Malignant cells multiply intensely at the expense of substances which pass through this dialysing membrane. They form aggregates, the histological structure of which is similar to the primary tumor, from which they were originated. The same results were obtained with crude extracts of mesonephros or with "conditioned" media, *i.e.* media in which mesonephros was cultured during several days and then carefully removed.

2. Pieces of fresh human surgical tumors were wrapped up in a vitelline membrane together with explants of chick mesonephros. In many cases, such as epithelial carcinomas of stomach and uterus, an abundant proliferation of the cancerous cells takes place at the expense of the mesonephros which becomes more or less completely invaded. The growing tumorous masses retain the same histological structures as in the *in situ* tumors.

The question arises whether the substances provided by the mesonephros are banal nutrients or specific growth promoting substances.

GENERAL SCIENTIFIC MEETINGS

AUGUST 28–31, 1961

Abstracts in this section (including those of Lalor Fellowship reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Molybdenum uptake of marine plankton algae. ROBERT J. BARSDATE AND ROBERT R. L. GUILLARD.

Molybdenum uptake by three species of marine planktonic algae was investigated using Mo-99 as a tracer. *Coccolithus huxleyi* (BT-6) and *Cyclotella nana* (clone 13-1) were isolated from the Sargasso Sea; *Synechococcus* sp. and *Cyclotella nana* (clone 3-H) are estuarine forms. All were grown in pure culture at 22° C. and 2000 lux in enriched sea water containing both nitrate and ammonia, and buffered to pH 8 with "Tris." The total molybdenum content of the medium was varied from 15 to 40 μg./l. (1.5 to 4 times the Mo concentration of Woods Hole Harbor water).

C. nana was grown in Mo-99-tagged media for about 7 cell divisions. Cell counts and molybdenum assay were then made, from which a concentration factor of *ca.* 6.5 was established. Labelled algae in darkness maintained their molybdenum concentration without change for 24 hours. There was no significant difference between the oceanic and estuarine clones.

When *C. nana* cells were exposed in darkness to an increase in molybdenum concentration of the medium, they equilibrated completely (to a concentration factor of 6.5) in four hours, after which there was no change. However, in illuminated flasks the addition of molybdenum resulted in an initial uptake exceeding that corresponding to a concentration factor of 6.5. After 12 hours the concentration factor was again 6.5. The molybdenum uptake by chloroform-killed *C. nana* cells was 70% that of living organisms (at 40 μg./l. of molybdenum).

The coccolithophoride, *C. huxleyi*, with a cell volume 2.5 times that of the diatoms, had a concentration factor of 4.8. *Synechococcus* sp., with a cell volume *ca.* 10^{-3} that of the diatoms described, had a concentration factor of about 70.

This study constituted one of of the staff-student research projects of the Marine Ecology Course (NIH Training Grant 2G–535) and was supported in part by a NSF grant.

Formation and possible function of the "secretory packets" of the starfish tube foot. ALFRED B. CHAET AND DELBERT E. PHILPOTT.

Further studies were carried out on the secretory structures found near the walking surface of the tube foot of *Asterias forbesi*. The "secretory packets" were seen with the electron microscope to be formed intracellularly in an area proximal to the bottom surface of the tube foot where the "packets" were released to the outside. In most cases the cytoplasm of the cell consisted mainly of endoplasmic reticulum, a Golgi apparatus, and associated mitochondria. Within these cells, several "secretory packets" generally were being formed. It appeared that a clear area first formed in the cytoplasm, which then contained a "secretory packet." This newly formed elliptical "packet" had darkly staining (osmophilic) ends between which were attached 50 or so fibers (200 Å diameter). During formation the osmophilic ends of the "packet" were attached to the rest of the cell by cytoplasmic strands, thus holding the "packet" in the center of the small clear area.

Attempts to follow the individual paths of the "secretory packets" from their intracellular origin to the secretory surface at the base of the tube foot have been unsuccessful, due to the tortuous nature of the long narrow channels.

In an effort to further the hypothesis that these "packets" functioned as a "glueing" mechanism, sections of the tube foot were stained by the P.A.S. technique. Observation with the phase contrast microscope demonstrated the "secretory packet" to be P.A.S.-positive, further suggesting their function as an adhering mechanism. These structures were not observable in the light or phase microscope until stained by the P.A.S. method.

Supported by grants from the National Science Foundation (G–8718) and the National Institutes of Health (A–3362).

Blood coagulation and fibrinolysis in the smooth dogfish. RUSSELL F. DOOLITTLE.

The hemorrhagic nature of elasmobranch blood can be remedied *in vitro* by the presence of unphysiologically large concentrations of calcium ions. In a milieu containing 50–75 m*M*./L.

calcium, dogfish blood plasma generates thrombin activity equivalent to mammalian systems maintained at physiological calcium levels. Under such conditions, other phases of the event become greatly exaggerated. Fibrinolysis occurs at a rate proportional to thrombin generation, and complete clot dissolution can be effected within 60 minutes of clot formation. Concomitantly, an intense serum inhibition develops which will prevent clotting when added to similar dogfish plasma systems. The inhibition is thermo-labile, partially dialyzable, and not reversed by toluidine blue. The site of inhibition is the thrombin-fibrinogen interaction or the subsequent polymerization of fibrin monomer units. If serum is separated from the fibrin clot before fibrinolysis occurs (*e.g.*, 30 minutes after clot formation), the inhibition does not develop, indicating that the fibrinolytic breakdown products are instrumental in the serum inhibition. Thrombin activity in such serum is neutralized as usual, however, suggesting two separate control devices. Comparisons of the ability of the generated thrombin to clot dogfish fibrinogen with its power to split the artificial substrates, tosyl-L-arginine methyl ester (TAMe) and L-lysine ethyl ester (LEE), showed that fibrinogen clotting power is neutralized much more rapidly than is the esterase activity. Other studies with TAMe and LEE suggest other esteratic or proteolytic events before and after clot formation which are distinct from the ability to clot fibrinogen. These include an initial sharp peak for TAMe esterase activity before the generation of thrombin and a slight increase in LEE esterase activity after clot formation.

This study was aided in part by a grant from the ONR Research Contract No. 1497.

In vivo and in vitro recovery of irradiated gametes of Arbacia punctulata. PATRICIA MCCLEMENT FAILLA.

It is known that exposure to ionizing radiation of either sperm or eggs of *Arbacia punctulata* delays the time of first cleavage of the fertilized egg. The magnitude of cleavage delay decreases exponentially with the time the irradiated eggs remain in sea water before fertilization (*"in vitro* recovery"). No such recovery occurs in irradiated sperm. Use of the electrical stimulation method to procure gametes has permitted investigation of the recovery process in gametes remaining inside irradiated urchins for periods of time up to two weeks (*"in vivo* recovery"). It was found that some recovery occurs in these eggs but at a rate about 100 times less than in the *in vitro* case. After about eight days no further recovery is detectable, but a slight cleavage delay persists. As in the *in vitro* case, no significant recovery was found for sperm *in vivo*. Also the ability of irradiated eggs to recover *in vitro* is no longer present when the eggs have remained in the animal more than four hours after irradiation.

It was thought that the slower recovery rate *in vivo* might be due to a lower metabolic activity of the eggs while in the ovary. The *in vitro* recovery process was followed, therefore, with the metabolic activity of the eggs reduced by keeping them in sea water in a nitrogen atmosphere for an hour after irradiation. Preliminary tests indicate that this recovery process is not affected by marked decreases in the oxygen tension. On the other hand, irradiation of the eggs in a nitrogen atmosphere caused a considerable reduction in the cleavage delay. The rate of subsequent *in vitro* recovery in air was reduced as well.

Absorption, accumulation and loss of radioactive scandium by marine macroalgae. JOHN GUTKNECHT.

Uniform discs of freshly collected *Ulva lactuca* and *Porphyra umbilicalis* were exposed to 20 uc./L. concentrations of high specific activity Sc^{46} in initially sterile and strongly buffered pH 8.0 sea water solutions in the light and the dark. Radioactivity of the algae was measured directly in a well scintillation counter.

Uptake-time curves were characterized by a roughly hyperbolic rapid absorption phase, followed by a more linear accumulation phase. In 48 hours the light *Ulva* absorbed 35% more Sc^{46} per unit weight than the dark, while the light *Porphyra* took up 15% more than the dark. High wet weight concentration factors were observed for both species, 2600 and 1500 times the external concentration in *Ulva* and *Porphyra*, respectively. Adsorption accounted for 40–60% of the total uptake as estimated by the intercept of the line of regression of the linear

part of the time curve. Thalli transferred after 48 hours from light to dark radioactive media showed a definite retardation of the Sc^{46} uptake rate. Algae killed with ethylamine absorbed more scandium than live material.

Loss of Sc^{46} was measured in buffered sea water in the light and the dark. Both species displayed a slow linear loss of scandium, less than 1% per hour. The light and dark algae showed no difference in loss rate.

Research supported by NIH Grant 2G-535 (MBL Ecology Training Program).

Properties of a newly isolated bacteriophage of luminescent bacteria. J. W. Hastings, A. Keynan and K. McCloskey.

Phage was isolated from a squid incubated with inoculations of marine mud and four strains of luminous bacteria. Phage containing samples were obtained by filtration and by treating with chloroform. Typical bacteriophage plaques were produced when tested with either *A. fischeri* or strain 60L (isolated from the Pacific Ocean by C. B. Van Niel) as host. The phage showed clear host specificity; with five locally isolated bacteria strains and one from Jamaica no plaques were observed. However, various "dark" mutants of the sensitive strain were susceptible to infection.

Plaques developed well in soft agar at temperatures between 20° and 30° C. The plaques always showed a turbid center, suggesting that the phage is a temperate one. In older plaques the phage-resistant bacteria completely overgrew the cleared areas, to an extent such that it was difficult to visualize the plaques. Observed in the dark, however, the formerly phage-cleared areas could be seen as non-luminescent spots on the background of the luminescent host strain. From this it was concluded that the phage-resistant cells growing in the center of the plaques were non-luminescent. "Dark" bacteria isolated from the center of the plaques exhibited the following properties which remained stable through several single colony isolations: (1) Ten of the fifteen isolates emitted light not visible to the eye but photometrically detectable; the others were intermediate in brightness. (2) All isolates were resistant to phage infection. (3) After being re-isolated four times from single colonies, all produced phage.

These observations support the view that these are lysogenic bacteria produced by phage infection, and that lysogenicity is in some way inversely related to light production.

The isolation and characterization of dark mutants of luminescent bacteria. A. Keynan and J. W. Hastings.

Numerous authors (Beijerinck, Van Niel, McElroy) have noted that dark mutants of luminescent bacteria accumulate in cultures. Biochemical studies of different dark mutants could yield information concerning the genetic and physiological control of bioluminescence. The present study is concerned with conditions under which dark mutants are found, their frequency and selection, and their physiological properties. Three strains were used in this work; (1) *Achromobacter fischeri*, (2) a strain isolated by Van Niel (L-60), and (3) a strain with a low temperature optimum isolated locally (W-14). From cultures started from a single bright colony, maintained on solid media, no dark colonies (out of several thousand) were seen; from similar cultures maintained in either minimal or complete liquid culture for extended periods (three days to three weeks), many dark colonies were observed upon plating out. From cultures maintained at high temperatures (up to 37°) a high proportion (30%) of dark colonies were found. Fifty-one dark mutants have been isolated and studied. Although several isolates may be derived from the same parental mutant, many are clearly different in origin and in physiological characteristics. All of the mutants which are dark to the eye emit some small amount of light which may be detected photometrically; the different mutants vary enormously with respect to their ability to emit, the intensity of the dimmest mutant being 10^{-5} of the wild type. The quantity of dark cells found in cultures suggested that the conditions gave selective advantage to dark mutants. This possibility was tested and supported in experiments where different proportions of wild type and dark mutant were mixed. It will be of interest to see if conditions where the wild type has a selective advantage can be found. This might be an experimental approach for studying the possible competitive advantage of bioluminescence.

Recent observations on Botryllus schlosseri. ROGER MILKMAN AND SYLVIA BYRNE.

We have confirmed Bancroft's observation of tremendous morphological diversity in the offspring of individual colonies of *Botryllus schlosseri* and his finding that the more closely related two colonies are, the more likely they are to fuse vascular systems when contiguous. Oka and Watanabe's demonstration of vascular budding in *B. primigenus* has been extended to *B. schlosseri.* Sabbadin's demonstration of selfing has been repeated.

Outcrossing, shown experimentally by Sabbadin, must be common in nature, too, for the great genetic heterogeneity of individual colonies would not otherwise be present. Fertilization by sperms arriving over a period of time probably accounts for the continuous variation in developmental stages we find in individual colonies of this viviparous organism, in contrast to the well-known synchrony of budding. Embryos may be raised outside the colony from before gastrulation. Attempts at external fertilization have failed.

Numerous traits, stable in asexual reproduction, have been characterized. Most of these depend upon the nature, quantity, and distribution of pigments; they are components of the colonies' over-all color patterns, of which there must be thousands. Inbreeding should permit development of homozygous strains to extend the recent observations of others on the genetic control of colony fusion and of pigmentation. *B. schlosseri* is notable for the unusual degree to which its genetic diversity is visibly obvious.

This work supported by a grant from NIH (Milkman) and an NSF Cooperative Summer Fellowship (Byrne).

Cortical granule extrusion during maturation of the egg of Asterias forbesi. ARTHUR K. PARPART.

The cortical granules of immature and mature eggs of *Asterias forbesi* were observed by television microscopy under conditions where individual cells were exposed to 1 *M* solutions of glycerol, erythritol, d-xylose, glucose or sucrose. In no case did these egg cells alter in volume, therefore these compounds do not enter the egg in the time period of observation, one hour.

The cortical granules of these eggs, however, reacted quite differently, dependent on the stage of maturation. The cortical granules of immature eggs showed no change, when exposed to the above solutions. The mature eggs exhibited a definite and violent explosion of their cortical granules in 1 *M* glycerol, erythritol or d-xylose; while no such explosion occurred in either 1 *M* glucose or sucrose. Prior to first polar body formation no cortical granules exploded under these conditions. Within a half hour after polar body formation all of the mature eggs had cortical granules which exploded, and showed membrane elevation.

These experimental observations suggest that the cortical granules of immature eggs are in the egg cytoplasm and that during the maturation process these cortical granules are extruded through the egg's plasma membrane and come to lie between it and the vitelline membrane. This is indicated by the fact that the polyhydric alcohols and sugars used cannot penetrate the plasma membrane. On the other hand a penetrating alcohol, ethylene glycol, caused the explosion of the cortical granules in both immature and mature eggs, and subsequently cytolysis. The fact that the above 1 *M* solutions caused an explosion of the cortical granules indicates that the granule's membrane must be permeable to the polyhydric alcohols and d-xylose but not to glucose and sucrose.

The α, β-unsaturated ether (plasmalogen) content of the tissues of several mollusks. MAURICE M. RAPPORT.

It was recently shown that the lipids of a number of marine invertebrates contain high concentrations (10 to 15%) of plasmalogens having the α, β-unsaturated ether structure (Rapport and Alonzo, J. Biol. Chem. **235**: 1953, 1960). Comparative studies have now been made of the lipids of individual tissues from 8 species of mollusk including 6 pelecypods (*Mya, Mytilus, Ostrea, Pecten, Spisula, Venus*), one gastropod (*Busycon*), and one cephalopod (*Loligo*). The α, β-unsaturated ether content of 5 or more tissues from each of these species was determined with respect to two reference bases: tissue dry weight and total lipid weight.

Among the pelecypods it was found that gill tissue contains the highest plasmalogen concentration, attaining values as high as 50 μmoles per gram dry weight and over 400 μmoles per gram of lipid (about 30% by weight). These values compare with those found in mammalian (rat) brain, the mammalian organ in which plasmalogens are most concentrated, namely, 60 μmoles per gram dry weight and 140 μmoles per gram of lipid. High plasmalogen concentrations are found in most pelecypod tissues, the lower values usually being associated with tissues of low lipid content (adductor muscle). Only crystalline style (*Venus, Pecten*) was found to be devoid of plasmalogen. In *Loligo*, a species in which plasmalogen concentrations are rather low, highest values were also observed in gills. In *Eriocheir*, the posterior gills, a salt-concentrating organ, were found by Dumont (Arch. Internat. Physiol. **66**: 373, 1958) to contain a high concentration of plasmalogen phosphatide, and this led him to suggest the possibility of a role for this lipid in ion transport. The findings in this study of mollusk tissues indicate that elevated plasmalogen concentrations may be a general characteristic of gills of all species, and are consistent with the suggestion that plasmalogens are involved in some type of transmembrane transport process.

Supported by N.I.N.D.B. research grant B–1570, U. S. Public Health Service.

Studies on the binding site of pinocytosis inducers. RONALD C. RUSTAD AND LYNNE C. RUSTAD.

Pinocytosis is a method of transport in which the formation of membrane-limited vacuoles is dependent on temperature and aerobic respiration. The first step in this process involves the attachment of inducers to the cell surface. In *Amoeba proteus* basic dyes act as inducers. These dyes compete with proteins for the same surface sites. The molecular orientation of the binding sites can be demonstrated by the birefringence colors of the attached dye. The dye colors obscure the natural surface birefringence; hence, the attachment is in immediate proximity to the major oriented component in the surface. Since proteins, which would not cross the permeability barrier, act as inducers, the binding region must be located outside the cell membrane.

The Alcian blue staining of sections of amoebae indicates that the extracellular coat is an acid mucopolysaccharide. This conclusion is further substantiated by the demonstration *in vivo* of alpha metachromasia when toluidine blue is used to induce pinocytosis.

This work suggests that pinocytosis inducers become attached to highly oriented sites in the extracellular acid mucopolysaccharide coat of the amoeba.

Culture of gonads and neural gland of Molgula manhattensis on various natural and synthetic media. PHILIPPE SENGEL AND MADELEINE KIENY.

The gonads and the neural gland were obtained from immature and mature animals. The gonads, including testes and ovaries, were explanted, together with the lateral wall of the body to which they are attached. The neural gland was cultured together with the brain and the vibratile organ.

Several media were tested as to their ability to provide adequate conditions for the survival of the organs *in vitro*. Some media contained 7 volumes of a 1% agar saline solution, 5 volumes of sea water and one volume of either sea water (medium M), chicken plasma, horse serum (medium HS), chick embryo extract, or Parker's solution no. 199. Other media were made of 7 volumes of the agar saline solution and either 6 volumes of horse-shoe crab serum or three volumes of sea water and three volumes of horse-shoe crab serum.

Immature gonads were associated on media M and HS with neural glands, including brain and vibratile organ, obtained from both juvenile and adult animals. In some cultures, however, the gonad was associated with the neural gland alone.

The explants were cultured for seven days and survived throughout this period. In most cases, the vibratile activity of the ovarian cavity, the spermiducts and the vibratile organ continued *in vitro* until the end of the cultivation period. The explants mostly maintained their organotypic organization, and very little outgrowth of cells occurred.

The histology of the cultured gonads and the possible action of the neural gland on their differentiation will be studied and described ulteriorly.

Effect of temperature, salinity, and food intake on the excretion of Zn^{65} in small marine fish. JUDITH SHULMAN, I. LEHR BRISBIN AND WALTER KNOX.

Fish of three species were tagged by feeding *Venus* meat soaked in $Zn^{65}Cl_2$ solution of approximately 1 μc./ml. concentration, and biological half-life ($Tb_{\frac{1}{2}}$) of assimilated isotope determined by making periodic whole-body counts in a well counter over a 15–20-day period. Fish were kept in clean tanks with running water or frequent changes of water.

Oxygen consumption measurements were also made on two species, using the Winkler method.

At 20° C. and 35‰ salinity, $Tb_{\frac{1}{2}}$ was 13 days for *Menidia menidia* as compared with 58 days for *Fundulus heteroclitus;* the metabolic rate of the former is about two times that of the latter. For *Fundulus,* $Tb_{\frac{1}{2}}$ was 75 days when kept at 10° C. and 35 days at 30° C., suggesting an inverse relationship of environmental temperature and excretion rate.

A preliminary experiment showed that *Fundulus's* $Tb_{\frac{1}{2}}$ was not different at 8‰ and at 35‰ salinity, indicating that gill salt excretion at high salinity does not materially increase the exchange of zinc.

No effect of varying food intake could be demonstrated. Zn^{65}-tagged specimens of *Tautogolabrus adspersus* at 20° C. and 35‰ salinity averaged a $Tb_{\frac{1}{2}}$ of 44.5 days, at food levels varying from 153.7 to 58.1 cal./g. fish/day, over a 17-day period. $Tb_{\frac{1}{2}}$ of one specimen of *T. adspersus,* which was starved for the 17-day period, was 59 days.

Research supported by N.I.H. grant 2G–535 (M.B.L. Ecology training program).

Aminopterin effects on Arbacia punctulata development. RICHARD G. SKALKO.

Aminopterin, a potent inhibitor of folic acid metabolism, produces a variety of developmental abnormalities in mammalian embryonic systems. This study was undertaken to determine whether this compound also interfered with the normal development of the sea urchin, *Arbacia.*

Eggs and sperm were obtained from mature *Arbacia* by mild electrical shock. Aliquots (100–300) of eggs were added to stender dishes containing the anti-metabolite dissolved in filtered sea water, either 30 minutes after fertilization or at the blastula stage. They were allowed to develop in a constant-temperature incubator (19° C.) and observations were made during cleavage, at hatching, at pre-pluteus and mature pluteus stages.

Exposure at fertilization over a range of doses (200-50 μg./10 ml. filtered sea water) produced no visible effect during cleavage. Beyond this stage, however, the effects were dosage-dependent. At 200 μg./10 ml., the eggs rarely became free-swimming blastulae and were killed at that stage. Eggs treated with 100 μg./10 ml. hatched normally but never developed beyond the blastula stage. They exhibited a characteristic circular swimming pattern and survived 10 to 14 hours beyond the 200 μg.-treated eggs. Those treated with 50 μg./10 ml. were completely inhibited as pre-plutei.

When the eggs were exposed to the same doses of aminopterin at the blastula stage, a different range of effects was observed. Those exposed to 200 μg./10 ml. developed into pre-plutei and rarely into immature plutei. Those treated with 100 μg./10 ml. developed into pre-plutei and immature plutei, while those treated with 50 μg./10 ml. developed into mature, immature and pre-plutei.

The results indicate that the effects produced by aminopterin are both dosage-dependent and time-dependent, the embryos being most sensitive during cleavage stages. It appears that, during cleavage, the anti-metabolite is interfering with a metabolic process that is essential for further development beyond the blastula stage.

Difference in chlorophyll a content of Cyclotella caspia cells grown on nitrate and uric acid. MARGARET J. WALDREP AND R. R. L. GUILLARD.

We determined the chlorophyll *a* content per cell and per unit of cell volume of *Cyclotella caspia* (clone 10–5) grown in pure culture in enriched sea water having either nitrate (100–400 μM) or uric acid (25–100 μM) as nitrogen source.

At the higher concentrations of either nitrate or uric acid the chlorophyll remained relatively high, varying from $5.1–8.0 \times 10^{-7}$ μg./cell, or from $3.8–6.9 \times 10^{-9}$ $\mu g./\mu^3$ of cell volume.

At the lower concentration of nitrate the chlorophyll *a* was lower, varying from $1.1–3.3 \times 10^{-7}$ μg./cell or from $1.1–2.8 \times 10^{-9}$ μg./μ^3 of cell volume. At the lower concentration of uric acid the chlorophyll remained high (4.5×10^{-7} μg./cell, or 4.2×10^{-9} μg./μ^3) during early growth of the culture but then fell in one day to 2.6×10^{-7} μg./cell or 2.2×10^{-9} μg./μ^3 of cell volume.

All observations are consistent with the hypothesis that availability of nitrogen rather than the specific nature of the nitrogen source is responsible for the variation in chlorophyll *a* content. However, the availability of nitrogen is influenced both by the nature of the source and its concentration.

C. caspia can utilize all the nitrogen atoms in the uric acid molecule (at 25 μM). Photosynthesis per cell, determined by C^{14} uptake, was about 30% greater when cells were supplied uric acid (100 μM) than nitrate (400 μM).

Active transport of oxygen into the eye of fish. JONATHAN B. WITTENBERG AND BEATRICE A. WITTENBERG.

The transport of oxygen against a concentration gradient has heretofore been described in only a single organ, the swimbladder of teleost fishes. M. Biot, in 1806, established that oxygen could be brought into the swimbladder against a pressure gradient of over 100 atmospheres. A century later W. N. F. Woodland, and J. S. Haldane showed that gas secretion was accomplished by a complex of two organs—a gas gland and a rete mirabile. The rete consists of a regular and intimate intercalation of venous and arterial capillaries, which follow parallel courses and carry blood to and from the gas gland. An entirely similar rete mirabile supplies blood to the choriocapillaris underlying the retina of teleost fishes. The pigment cell layer of the retina may be regarded as analogous to the gas gland. It occurred to us that the rete mirabile-pigment epithelium complex might build up a large tension of oxygen behind the retina, which would provide a pressure head for diffusion to supply the vigorous oxygen demand of the relatively avascular retina.

We have used a polyethylene-covered polarographic oxygen electrode to measure the oxygen tension in the vitreous humor immediately in front of the retina. Eight species of teleost with well developed retia exhibited oxygen tensions greatly in excess of that in air (160 mm. Hg); tensions ranging from 400 to over 1000 mm. were commonly encountered. Two species of teleosts with poorly developed retia exhibited tensions not too different from air; three elasmobranchs and two species of teleosts, which have no rete, displayed oxygen tensions at the retina substantially less than that of air.

Our finding establishes a second instance of oxygen secretion against a large concentration difference. The parallel between the degree of development of the rete and the oxygen tensions measured at the retina constitutes strong evidence that the choroid rete is an important member of the oxygen-secreting complex.

PAPERS READ BY TITLE

Cytochemical observations of oxidative activity in the developmental stages of Chaetopterus pergamentaceus, using succinate and DPNH as substrates. M. JEAN ALLEN.

Activity of DPN diaphorase and succinic dehydrogenase was demonstrated in whole mounts and serial sections following incubation with DPNH-Nitro-BT and succinate-Nitro-BT. At the germinal vesicle stage, using DPNH as substrate, purple to blue granules of diformazan (indicating DPN diaphorase activity) are concentrated in a perinuclear ring. From the nuclear membrane a decreasing gradient of activity extends into the cytoplasm surrounding the endoplasmic spherules. As the germinal vesicle breaks down, activity becomes concentrated in a broad crescent-shaped area (side view) of non-spherular endoplasm and residual substance around the spindle. At interphase of 2- and 4-cell stages, diformazan granules are present as a perinuclear ring and as a centro-peripheral gradient in the endoplasm. Perinuclear rings of activity were observed in later cleavage stages. In early blastulae, activity was clearly demonstrated only in the surface blastomeres. Oxidative activity in swimming larvae of 8 to 13 hours is primarily in the ectodermal cells (yolky endoderm showed no activity), as is

true in 24-hour trochophores except for the large mesotrochal cells which show no activity. For 1½- to 7-day trochophores, results are similar except that two small surface mucous glands at the level of the intestine show comparatively high activity.

Activity of succinic dehydrogenase was much less marked. Incubation time in succinate was 2½ to 3 hours instead of 7 to 10 minutes (DPNH incubation time) and even then, evident particularly in later stages, a pink rather than a purple to blue coloration may be noted in stages observed *en masse* as well as microscopically. The faint pink coloration was difficult to localize. The mucous cells of 1½- to 7-day larvae were not demonstrated following succinate incubation; this contrasts with the high concentration of oxidative demonstrated in these cells following incubation in DPNH.

Supported by NSF grant G–8775.

Techniques for obtaining, dissociating, and culturing cells and organs of Loligo pealii embryos. JOHN M. ARNOLD.

Some technical problems of obtaining and handling eggs and embryos of *Loligo pealii* were solved in the following manner.

It was possible to stimulate captive adults to lay eggs by suddenly exposing them to a naturally laid egg mass. This immediately resulted in both the males and females "investigating" the egg mass, mating, and egg-laying by the females. This is apparently a completely visual stimulus since an artificial egg mass made of water-filled polyethylene tubing would elicit the same response. Squid, in nature, have deposited fresh eggs on an egg mass hung from a piling after dark. In this way, the exact time of laying could be observed and very young stages of naturally laid eggs could be obtained.

It was found that dechorionated embryos, isolated organs, and single cells could be cultured in whole adult blood. This corroborates similar results by H. Hibbard and H. Hamilton (personal communication). Sterile culture techniques were used and antibiotics were added to prevent contamination. These embryos and organs appeared to develop normally.

It was possible to dissociate the cells of these embryos and to obtain solid aggregates within 15 minutes. These cells were dissociated in two different ways. In the chorion they were dissociated by ten minutes of violent shaking in sea water at pH 5. This treatment removes the jelly coating as well. In culture the cells were dissociated by crushing the embryo and mechanically selecting the single cells. Aggregates from both treatments survived up to 72 hours. As of yet, histological examination has not been made.

A longer paper giving further details is planned.

Observations on the mechanism of cellulation of the egg of Loligo pealii. JOHN M. ARNOLD.

Cleavage of the zygote of *Loligo pealii* leads to the formation of a blastoderm in about 12 hours. The blastoderm expands over the surface of the yolk so that the whole embryo is eventually composed of a surface layer of cells and a central yolk mass. The mechanism by which the yolk mass becomes covered by cells was the subject of this investigation.

Adult squid were kept in a deep sea-table and stimulated to mate and lay eggs. Eggs that were about one-half covered with cells were dechorionated and placed in depressions in a wax-bottomed watch glass containing sterile sea water. The embryos will live for about 24 hours under these conditions. The surfaces of the cells were marked with carmine powder, carbon particles, and/or Nile blue sulfate. Marks were made in the region above, at, and below the margin of the cellulated area. The margin of the cellulated area progressed further down the surface of the yolk and passed under the marks, but in no case did any major movement of the marks take place, although minor rearrangements did occur. This indicates that cellulation of the zygote takes place by peripheral cell division rather than by epiboly.

To negate the possibility of a non-moving surface coat, Nile blue sulfate marks were made on the eggs in addition to the particulate marks. These marks did not move in relation to each other. When the embryos at this stage were placed in 1:10,000 colchicine, cleavage was stopped and the cellulation of the zygote also stopped. Direct observations support this hypothesis also.

Evidence of a marginal zone of proliferation was found in aceto-orcein squashes which show a higher rate of mitosis at the edge than in the center of the blastoderm.

Responses of the mud snail, Nassarius, to experimental reversals in direction of very weak magnetic fields. FRANKLIN H. BARNWELL AND H. MARGUERITE WEBB.

Snails emerged from a magnetic-south-directed aluminum funnel-shaped corral. The mean amount of turning was expressed in terms of angular deviation from south (designated 0°) as the snails reached a point 3 cm. from the exit. The field into which the snails emerged was asymmetrical, black to left and white to right; the mean path of the snails preferred the white. To avoid the known daily fluctuation in mean path, all series were run between 8 AM and 12 M. Each daily experiment comprised assaying mean snail paths when the H component of magnetism was experimentally reversed to produce north-directed fields of 0.04, .1, .2, .4, .8, 2, 5, and 10 gausses. Control runs in the earth's field alone preceded and followed each pair of experimental fields. Each experiment comprised moving up the series with five paths in each, and then moving back down the series. Differences between experimental and adjacent control paths were determined. A significant difference ($P < .02$) in mean path from the controls was found only for the reversed 0.2 gauss experimental field, with only slightly weaker response in the 0.1 gauss field. The 0.1 and 0.2 gauss field effects exhibited essentially a unimodal distribution of values while the remainder were more or less bimodal, suggesting the fields to be inducing right *or* left turning. A study of the structure of the regressional relationship between mean path of all snails in a given series and magnet response revealed that when the path was more weakly (+2.5° to +12.5°) or more strongly (+15.5° to +24.5°) positive than near average (+ 12.5° to + 15.5°) the 0.2 gauss field was turning them highly significantly ($P < .001$) to the right; the .04 gauss field induced left-turning. The controls displayed a substantial lunar tidal fluctuation ($P < .001$) in mean path.

This study was aided by a contract (1228–03) with the Office of Naval Research and by grants from the National Science Foundation (G15008) and National Institutes of Health (RG–7405).

In vitro incorporation of C^{14}-labeled amino acids into goosefish islet tissue proteins. G. ERIC BAUER AND A. LAZAROW.

Islet tissue (2–6 mg.) was incubated for two hours in a modified Krebs-Ringer bicarbonate buffer containing leucine-C^{14} ($2.1 \times 10^{-3}M$) plus valine-C^{14} ($3 \times 10^{-3}M$). Specific activities were 6–10 mC./mM. Proteins were precipitated and washed with 5% trichloroacetic acid, extracted with 95% ethanol, ethanol-ether, and ether. Aliquots of both the alcohol-soluble fraction (ASF) and the final insoluble protein were removed and the radioactivity determined. When I^{131}-labeled insulin was added to islet tissue as a marker, 80–90% of the radioactivity was recovered in the ASF.

The average two-hour C^{14} incorporation into the ASF in 33 samples was 5720 cpm/10 mg. of tissue. Incorporation of C^{14}-amino acids into the ASF increased progressively during 9 hours of incubation (3900 cpm at one hour, 15,000 at four hours, and 23,500 at 9 hours). The incorporation was oxygen-dependent (up to 90% inhibition by anaerobiosis). When the amino acid concentration in the incubation medium was decreased ten-fold the counts incorporated into the ASF were decreased by only 50%. The addition of glucose (at concentrations of 100 and 200 mg. per 100 ml.) likewise decreased the counts in the ASF by 50%.

Further studies on purified ASF (dried, ether-extracted, dissolved in acid alcohol, and reprecipitated by acetone) using I^{131}-insulin as a marker, indicated that the amount of insulin precipitated by acetone was dependent upon the amount of unlabeled insulin present. Purification of the ASF obtained from the amino acid incorporation experiments indicated that 65–89% of the counts were precipitated by acetone. The average specific activity of the purified ASF was 129,000 cpm/mg. protein (*i.e.*, three times greater than that of the insoluble protein residue). These studies suggest that amino acids, added to islet tissue *in vitro*, are incorporated into insulin.

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The ionic factors in the electrogenesis of the electrically inexcitable and electrically excitable membrane components of frog slow muscle fibers. PETER BELTON AND HARRY GRUNDFEST.

The depolarizing electrogenesis of frog slow muscle fibers is due to postsynaptic potentials (psp's) generated in electrically inexcitable synaptic membrane by neural volleys or by cholinomimetic agents. However, the fibers also possess a membrane component which responds to sufficiently large depolarizing stimuli, which may be the psp's themselves, or depolarizations from intracellularly applied currents of sufficient magnitude to cause depolarization above the threshold for this electrically excitable response. The response is a hyperpolarizing (or repolarizing) electrogenesis, with which is also associated increased conductance of the membrane. The electrogenesis of the synaptic membrane appears to be due to increased conductance for sodium and potassium, as appears to be also the case in frog twitch muscle fibers and *Raia* electroplaques. Removal of chloride from the bathing medium, which inverts the normal hyperpolarizing inhibitory psp's that operate through increased chloride-conductance and which causes prolonged "spikes" by the chloride-conductance operated electrically excitable rectification of *Raia* electroplaques, does not affect the depolarizing psp's of the frog slow muscle fibers. Removal of chloride also does not affect the electrically excitable responses of the muscle fibers, while removal of potassium increases the hyperpolarization of the fibers. Thus, the electrically excitable electrogenic response of the frog slow muscle fibers appears to be due to potassium-activation.

Electron microscopic observations on the effects of homologous egg water on echinoderm spermatoza.. MAURICE H. BERNSTEIN.

Egg substances in contact with spermatozoa of the same species are credited with causing the formation of a filament from the substance of the acrosome. This acrosome reaction is sometimes accompanied by an agglutination of the spermatozoa. An electron microscopic examination of three species of echinoderms has provided a morphological basis for these egg water reactions and some further insight into the nature of acrosome filament formation and the mechanism of sperm agglutination.

The spermatozoa of *Arbacia punctulata, Echinarachnius parma,* and *Asterias forbesii* share a relatively simple morphology, being made up of an acrosome, head, middle piece, and flagellum. Sea urchin and sand dollar spermatozoa have a simple acrosome with a single, membrane-limited apical granule set in an anterior depression of the head. The starfish acrosome has a number of components and is of considerably greater complexity. In all three species the middle piece is dominated by a single ring-shaped mitochondrion.

The response of spermatozoa to treatment with egg water can be considered as a two-step reaction. In the first phase, the substance of the acrosome loses its structured appearance, apparently undergoing a solubilization or hydration. In the second phase, contact with any one of a variety of relatively non-specific surfaces will elicit the formation of an acrosome filament. These non-specific surfaces may include other eggs, glass, plastic films or particles.

The change in the appearance of the acrosome on egg water treatment is accompanied by an expansion or swelling of the middle piece. The cristae of the mitochondrion are disarrayed and partially disintegrated and the sperm membrane is distended to accommodate the increased volume of the middle piece. In agglutination, adhesion is mediated by contact with the surface of the swollen middle piece.

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The corneal endothelium of the scup (Stenotomus chrysops). LASZLO Z. BITO AND CLIFFORD V. HARDING.

The mammalian eye is known to have a sheet of endothelial cells completely covering the posterior surface of the cornea. This endothelium is known to have a very important

function in the metabolism of the cornea. The present studies demonstrate that there is a similar layer of cells in the scup eye and that this endothelium is a suitable tissue for *in vitro* studies of DNA synthesis. Whole-mount preparations of the posterior surface of the cornea were stained with Harris hematoxylin. Faintly stained nuclei were apparent in some areas, but for the most part, the more heavily-stained underlying stromal elements obscured the picture. This difficulty was overcome by making extremely thin preparations from corneas which had been stained according to the Feulgen procedure. Such preparations showed distinctly stained nuclei, quite evenly distributed over the entire posterior surface of the cornea. These nuclei showed a greater diversity in morphology than nuclei of similar preparations of mammalian (rabbit) corneal endothelium, and the number of cells per unit area is smaller than in the rabbit.

DNA synthesis was studied by autoradiography of endothelial preparations after the corneas had been incubated in teleost Ringer containing H^3-thymidine. Corneas incubated for 2–6 hours showed incorporation by a varying number of cells around the periphery of the endothelium. Similar incorporation can also be obtained after 24–48 hours of non-radioactive pre-incubation, indicating that these cells retain their DNA synthesizing ability *in vitro* for at least 48 hours. Corneas isolated and incubated 24–48 hours after a central penetrating needle injury showed marked DNA synthesis in the central area of the endothelium. An *in vitro* injury of the endothelial surface of freshly isolated corneas did not stimulate thymidine incorporation 24-48 hours after injury. Mitotic figures were not evident in any of the endothelial preparations.

Preliminary measurements of filter-feeding activity of the soft-shell clam, Mya arenaria, by use of radioactive algae. JOHN W. BLAKE.

Filtration rates (volume of water from which all particles are removed in unit time) of the soft-shell clam, *Mya arenaria*, were studied with single-species cultures of unicellular marine algae labelled with P^{32} (methods developed by Drs. T. R. Rice and W. A. Chipman, present work initiated while visiting the Radiobiological Laboratory, Bureau of Commercial Fisheries, Beaufort, North Carolina).

Data were obtained relating filtration rates to combinations of the following parameters: clam size (5 sizes, ½ to 2 inches shell length, 0.1 to 11.0 gms. wet meat weight, respectively), species and size of algal cells, algal population density, and salinity.

Preliminary findings indicate similarities between *Mya* and the hard-shell clam, *Mercenaria mercenaria* (data from Rice and Smith 1958, Smith 1958). Filtration rate of *Mya* appears independent of salinity (32 vs. 16‰), and algal population densities from 3×10^6 to 1.5×10^9 cells per liter (filtration is inhibited severely at 3×10^9 cells per liter). Filtration rate does vary with size or species of algal cells (2-inch clam filters *Nitzschia closterium*, $5 \times 30\,\mu$, at an average of 2.0 liters per hour, *Carteria* sp., $16 \times 19\,\mu$, at 1.6 L./hr., *Nannochloris atomus*, $2 \times 2\,\mu$, at 0.9 L./hr.), and also varies with clam size (2-inch clam filters *Carteria* at 0.15 L./hr./gm. clam meat, ½-inch clam at 3.0 L./hr./gm.).

While trends are similar, soft-shell clams, especially the smaller sizes, maintain an advantage over the hard-shell clams in the efficiency with which they are able to remove small particles, *i.e.*, *Nannochloris*, from the water. This may relate to the greater abundance of smaller sized phytoplankton species and cells in the more estuarine habitats of the soft-shell clam.

Supported by a grant from the Aquacultural Research Corporation, Chatham, Massachusetts.

Comparison of the osmotic activity of ocular fluids with that of arterial plasma in the dogfish. M. BLOETE, D. C. NAUMANN, H. S. FRAZIER, A. LEAF AND WILLIAM STONE, JR.

It has been previously demonstrated that the aqueous humor is slightly, but significantly, hypotonic to the venous plasma in the smooth dogfish (*Mustelus canis*). The aqueous is currently assumed to be secreted into the posterior chamber, circulated anteriorly through the pupil, and reabsorbed finally at the base of the iris. The observed hypotonicity, therefore, might result from secretion of an isotonic fluid into the posterior chamber with subsequent dilution by reabsorption of solute. To test this hypothesis, tonicity of fluid from anterior and posterior chambers, as well as of the vitreous body, was determined.

Minute droplets of fluid were obtained under oil from the chambers of the eye of lightly curarized dogfish. Arterial blood was collected from the caudal aorta. Osmolality of these fluids was determined with a Ramsey-Brown Osmometer. Duplicate measurements, agreeing within 1%, were made with a Fiske Osmometer when sample volumes were sufficient.

In eleven dogfish, the osmolality of aqueous humor averaged 963.7 ± 3.8 mOsm./kg. water (S.E. mean) while arterial plasma averaged 978.3 ± 2.8; the mean difference is 14.5 ± 3.8 and $p < 0.01$. This result substantiates the difference previously reported between aqueous humor and venous plasma. Additional measurements showed: arterial plasma ($n = 15$) averaged 981.3 ± 2.2 (S.E. Mean), anterior aqueous ($n = 15$), 960.7 ± 2.8, posterior aqueous ($n = 12$), 952.8 ± 3.7; and vitreous ($n = 6$), 948 ± 1.3. The mean difference between plasma and anterior aqueous was 19.9 ± 4.1 ($p < 0.01$) and between anterior and posterior aqueous 7.4 ± 1.9 ($p < 0.01$).

Thus, there is a concentration gradient: arterial plasma > anterior aqueous > posterior aqueous > vitreous. Hence, (1) this sequence is incompatible with the gradients expected from the original hypothesis; (2) this sequence could not result from entry of water through the cornea from the more hypotonic sea water: (3) no metabolite is present in an amount sufficient that its utilization by the retina could account for the osmotic differences.

In the teleost (*Stenotomus chrysops*) whose plasma is hypotonic to sea water, limited measurements indicate that ocular fluids are also hypotonic to plasma.

Organismic orientation relative to magnetic axes, in responses to weak magnetic fields. FRANK A. BROWN, JR. AND FRANKLIN H. BARNWELL.

The path of mud snails, *Nassarius*, initially directed in each of eight equally spaced angular relationships to the horizontal component of geomagnetism from 0° to 270° was determined between 1 and 4 PM every two to three days for the period, June 21 through August 18, 1961. The average paths of four groups of ten snails were obtained on each day, with five snails emerging at each orientation starting at 0° and five snails passing in the reverse order. The snails emerged into an unchanging asymmetrical field with black to left and white to right. A total of 1000 snail paths in each of the eight relationships was obtained. The snails, favoring the side of brighter illumination, displayed a maximum right-turning when magnetic north-directed and minimum when northeast (45°) directed. ($P_{diff} < .001$.)

Although the snails turned progressively more to the right as the angle increased from 45° to 270°, they turned significantly ($P < .005$) further left when the magnetic axis was at 45°, 135°, 225°, and 315°, than at adjacent parallel or right-angle positions. The effect of magnetic N, E, S, and W relationships was confirmed (north and east difference, $P < .001$) by experiments on alternating afternoons for the same period. That magnetism itself was in some manner involved was demonstrated by a parallel series of experiments in which the orientation of the snail chamber itself remained unaltered but a horizontal *5 gauss* field was rotated and control observations interpolated. A significant difference ($P < .02$) in response to the magnet as a function of magnetic orientation was observed, of the same general amplitude, but closely mirror-imaging the corresponding relationship in the earth's 0.2 gauss field. Other experiments established ($P < .005$) that the snails showed left-turning in response to 2- and 5-gauss vertical fields.

This study was aided by a contract (1228-03) with the Office of Naval Research and by Grants from the National Science Foundation (G-15008) and National Institutes of Health (RG-7405).

Lysergic acid and growth in Fundulus. JOSEPH A. BURKE AND JOSEPH C. ORLANDO.

Previous immersion experiments have shown that lysergic acid diethylamide (LSD-25) inhibits growth of eggs of *Fundulus heteroclitus*. To ascertain more precisely the developmental stage at which LSD exerts its greatest effect, eggs were placed in 100 microgram/ml. solutions of LSD-25, (1) during fertilization; (2) from the 2-cell stage (Op. 3) to blastula (Op. 8); (3) from Op. 8 to Op. 15 (closure of the blastopore); the eggs were then placed in pure sea water. LSD does not inhibit fertilization nor are there any apparent effects from exposure (1) or (2). Eggs treated as in (3) were noticeably retarded about Op. 19–20 when the

nervous system is being laid down. Mean heart rates for all days up to three days after hatching were: controls, 144 beats/minute; test embryos, 99/minute. However, unlike eggs grown from fertilization to hatching in LSD, these embryos gradually recover. Three to four days after hatching, test embryos look like controls and their pulse is the same. In another series of experiments, to determine the lasting effects of LSD, eggs at Op. 11 were placed in a 1 mg./ml. solution of LSD-25 for periods of 1, 4, 10, 24 and 72 hours, respectively, and then returned to sea water. Depending on time of exposure to LSD, all embryos showed some inhibition. First measurable heart rates averaged: controls, 120 beats/minute; 1-hour embryos, 108/minute; 4-hour embryos, 95/minute; 10-hour embryos, 87/minute; 24-hour embryos, 72/minute; 72-hour embryos, 69/minute. By two days after hatching, all embryos had recovered, except the 72-hour embryos which died before hatching due to severe abnormalities in the nervous and circulatory systems. In the 1-, 4-, 10- and 24-hour embryos, the melanophores were more dispersed and stellate in form than in the controls, indicating a still persistent effect of the LSD substance. Histological studies of preserved test embryos will be made to ascertain whether there is any damage at the cellular level.

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Organization of the cockroach respiratory center. JAMES CASE.

The isolated ventral nerve cord of the roach, *Blaberus craniifer,* continues to generate rhythmic volleys, judged to be respiratory motor since they appear only in the innervation of the respiratory musculature and are highly sensitive to pCO_2. In such preparations localized application of CO_2 to any ganglion intensifies and accelerates the rhythm. Contrasting with this widespread sensory capacity, the rhythm does not appear unless certain combinations of ganglia are present. Specifically required are the first abdominal ganglion and a minimum of either adjacent ganglion. Dissociated from ganglia anterior to the second abdominal, the cord remains highly sensitive to CO_2 but responds to it only with increased random spike activity. The respiratory control center would thus seem to function by rhythmically applied inhibition of tonic motor neuron activity in the remainder of the cord. Because single electrical stimuli applied at any point in the cord or to the roots of the segmental nerves interrupt a respiratory volley in progress and induce a series of ten or so brief volleys which gradually return to pre-excitation duration and rate, it appears that the normal respiratory rhythm also involves excitation of the inhibitory center by respiratory motor activity throughout the cord.

Effects of acids on an isolated insect respiratory center. JAMES CASE.

Under relatively normal conditions CO_2 has been considered to act as a respiratory stimulus in insects by means of some specific property other than the acidity it generates in solution. Experiments with the isolated ventral nerve cord of *Blaberus craniifer* show that this is not correct. Under the proper conditions, any of the thirteen weakly dissociated acids tested were able to induce respiratory rhythms in a completely reversible manner. These acids were not effective at the same pH so it may be concluded that hydrogen ion alone is not the governing factor. The more highly dissociated the acid the lower the pH had to be before respiratory volleys were generated. Thus, while unbuffered butyric acid saline was effective at pH 4.1, maleic acid solutions had to be carried to pH 3.6 before becoming effective. Further, the effective pH of carbonic acid-bicarbonate solutions varied uniformly with the bicarbonate concentration, from pH 5.0 for .001 *M* to pH 6.7 for .04 *M* bicarbonate. These observations are taken to imply a respiratory control mechanism sensitive to hydrogen ion concentration exists in the roach central nervous system, and that it is isolated by a barrier with preferential permeability to undissociated molecules.

Further studies on the gamete "shedding substance" from radial nerves. ALFRED B. CHAET AND ROBERT A. ROSE.

The presence of a gamete "shedding substance" extracted from starfish (*Asterias forbesi*) radial nerves by lysis in distilled water has been reported in a previous communication. Nerve

extracts, when first autoclaved and then dialyzed against large volumes of sea water, induced shedding of gametes (within one hour) in starfish receiving an intracoelomic injection (0.15 cc./gm.). These results suggested that the "shedding substance" was non-dialyzable. Further experiments, however, indicated that the "shedding substance" did indeed dialyze through Visking tubing prior to autoclaving, but did not dialyze if the extract was autoclaved prior to dialysis. Such results suggested that autoclaving allowed the "shedding substance" to complex either with itself, or with other material in the extract, thus changing its dialyzable properties. The active component ("shedding substance") in a distilled water extract diffused across a membrane if dialysis was carried out against sea water, but did not dialyze if the dialysis was carried out in the absence of salts. The "shedding substance" found in a dialysate of sea water was ether-insoluble and was destroyed by autoclaving, but was only partially inactivated by boiling for 20 minutes. The "shedding substance" can be released not only by lysing radial nerves in distilled water (1 cc./nerve), but was also released when nerves were exposed to ethanol for 30 minutes. The fact that the active component in starfish nerves was not follicle-stimulating hormone (FSH), luteinizing hormone (PLH), 4-aminobutyric acid, or DL 2-aminobutyric acid, was seen when injections of the above solutions failed to induce the release of gametes from ripe starfish.

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Mutarotase inhibition by urea and guanidine hydrochloride. AURIN M. CHASE AND HILDEGARD C. VON MEIER.

The activity of kidney mutarotase preparations was measured polarimetrically by determining the first order reaction rate constant from the change in optical rotation when alpha-D-glucose was converted to the equilibrium mixture of the alpha and beta forms in the presence or absence of the enzyme, and in the presence of the inhibitors. All experiments were done at 26° C. in pH 5.3, 0.05 *M* acetate buffer, phosphate buffer having been found to have itself a greater catalytic effect on mutarotation than acetate and to inhibit mutarotase activity.

Guanidine hydrochloride was about ten times as effective as urea, but an analysis of the data representing relative activity as a function of inhibitor concentration indicated that about four molecules of either urea or guanidine combined with one mutarotase molecule (or active site) to produce the inactive complex.

An analysis by the Lineweaver and Burk method showed that the inhibition by guanidine hydrochloride is of the competitive type. Mutarotase would therefore fit in the general category of enzymes considered by Rajagopalan, Fridovich, and Handler (J. Biol. Chem., 1961), who found that those with organic substrates were competitively inhibited by such substances as urea or guanidine.

Exploratory experiments indicate that the percentages of inactivation of mutarotase produced by various concentrations of guanidine are essentially the same whether the enzyme is catalyzing the mutarotation of alpha-glucose or of beta-glucose.

The time required for half-completion of the enzyme-catalyzed mutarotation of alpha-glucose increases as the initial concentration is increased, over the range from 2½ to 10%.

Supported in part by a grant from the National Science Foundation.

Zygote versus gamete irradiation in Arbacia. RALPH HOLT CHENEY AND CARL CASKEY SPEIDEL.

The damaging effects of x-ray irradiation of zygotes and gametes in *Arbacia* contrast sharply with those following 2537 Å ultraviolet irradiation. Extensive series involving graded doses of both types of radiation were studied with special reference to developmental delay and injury in the progeny during the first two days. X-ray dosages ranged from 1 to 128 kr. Ultraviolet exposures ranged from 30 seconds to 12 minutes.

X-ray effects. (1) A 1-unit dose to zygotes (irradiation begun one minute after mixing normal eggs and sperm) caused damage *equal* to or *greater* than a 1-unit dose to both gametes subsequently intermixed for fertilization. (2) A 1-unit dose to zygotes also induced damage

equal to or greater than an 8-unit dose to eggs alone which were then fertilized with normal sperm, or than an 8-unit dose to sperm alone which were then combined with normal eggs.

Ultraviolet effects. (1) In contrast with no. 1 above, a 1-unit ultraviolet dose to zygotes (irradiation begun one minute after intermixing normal gametes) caused *much less* damage than a 1-unit dose to both gametes. Furthermore, a 4-unit or even 8-unit dose to zygotes also caused less damage. An essentially similar result, though the contrast was not so great, was obtained from zygotes *shaken* throughout the irradiation period. (2) In contrast with no. 2 above, a 1-unit dose to *unshaken* zygotes caused damage about equal to a 2-unit dose to *unshaken* eggs alone; also a 1-unit dose to *shaken* zygotes caused damage about equal to a 2-unit dose tc activated sperm alone.

Pigment distribution in degeneration (recorded by time-lapse motion pictures). Strong x-ray or ultraviolet irradiation of zygotes, both gametes, or eggs alone often induced violent internal upheaval followed by localized massive pigment concentration. Strong irradiation of sperm alone, however, usually induced abnormally scattered, spotty pigment distribution.

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Further experiments on the role of the polar lobe region in embryonic determination in Ilyanassa. ANTHONY C. CLEMENT.

Through removal of the D macromere (and hence of the polar lobe region) from the egg of Ilyanassa at successive cleavage stages, the following types of partial eggs were produced: (1) ABC, (2) ABC + 1d, (3) ABC + 1d + 2d, (4) ABC + 1d + 2d + 3d, (5) ABC + 1d + 2d + 3d + 4d. All of the types produced partial larvae except the last, which gave rise to a complete veliger of reduced size.

Types 1 and 2 gave partial larvae of the sort obtained after removing the polar lobe at first cleavage. Larvae of type 3 were similar to the preceding except that occasional individuals bore a small external shell. Larvae of type 4 were much superior to the preceding, often showing an organized velum, paired eyes, foot, and well developed shell. Heart and intestine appeared only in larvae of type 5.

The difference in development of larvae of types 3 and 4 cannot be attributed solely to the presence or absence of the third micromere. In a different series of experiments the third micromere was removed from otherwise whole eggs; the resulting larvae showed foot deficiencies, but good development of eyes, velum and shell. The polar lobe region appears to play a role of induction or activation in the determination of certain larval organs.

Fertilizability and developmental capacity of germinal vesicle and non-germinal vesicle eggs of Hydroides. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

Batches of eggs shed by the female upon removal from the dwelling tube were examined. The germinal vesicle, though usually intact in the full sized egg, was often found to have broken down in a few eggs, and in some batches it had broken down in as many as 50% of the full sized eggs. When the germinal vesicle has broken down, an ill-defined clear area lies in the cytoplasm nearest the animal pole.

Eggs of both types were isolated, inseminated, and compared as to fertilizability and capacity to develop. Uninseminated controls of both types were isolated from the same batches. It was found that the egg can be fertilized in either the germinal vesicle or non-germinal vesicle condition. Inseminated eggs of both types can develop into normal trochophores. From some females the "non-germinal vesicle eggs" gave higher percentages of normal development than did the "germinal vesicle eggs." In other batches, both types of egg gave about the same percentages of normal development. None of the uninseminated control eggs underwent any real development whatsoever. However, the uninseminated non-germinal vesicle eggs eventually undergo a type of fragmentation which bears some superficial resemblance to cleavage.

It is suggested that the eggs in which the germinal vesicle normally breaks down soon after shedding are those which have most nearly reached the peak of their physiological maturity.

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Studies on the site of action of alloxan: lack of penetration into cells. S. J. COOPERSTEIN AND JAMES A. JACKSON.

As part of a project designed to determine the mechanism by which alloxan selectively kills the insulin-producing cells of the islets of Langerhans, we have previously compared the distribution of injected C^{14}-alloxan with that of C^{14}-mannitol, C^{14}-thiocyanate, C^{14}-inulin, and C^{14}-urea in islet and other tissues of the toadfish. Since the distribution of injected alloxan paralleled that of mannitol, inulin, and thiocyanate (compounds whose distribution is limited to the extracellular fluid), it was suggested that alloxan does not enter the cell but exerts its diabetogenic effect by acting on the beta cell membrane.

In the present study the distributions of C^{14}-alloxan and H^3-mannitol were measured simultaneously in the same fish; this procedure permits a more precise comparison between these two compounds than was possible when separate animals were used. A mixture of tracer doses of these two radioactive compounds was injected intravenously into toadfish; 7 fish were killed at 2 minutes after injection, 5 at 5 minutes, and 6 at 15 minutes. Samples of islet and other tissues were removed and the amounts of C^{14} and H^3 present were determined using the Packard Tri-Carb liquid scintillation counter.

The results showed that at all times following injection the relative amounts of C^{14}-alloxan and H^3-mannitol in islet, heart, muscle, gill, kidney, brain, and blood were the same as that in the injected mixture. Liver differed from the other tissues; it contained a relatively greater amount of mannitol, due to the penetration of this compound into liver cells. These findings support the thesis that alloxan does not enter the cell during the period when it is known to exert its diabetogenic effect, and that the beta cell membrane is a primary site of alloxan action.

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Comparison of serum-proteins from young and old Limulus. ARMANDO A. DE LA CRUZ AND ALFRED B. CHAET.

It has previously been reported that young horseshoe crabs (*Limulus*) exhibited a resistance to burn toxin which proved fatal to older animals. These findings suggested a comparative study of the serum-proteins between young and old horseshoe crabs (*Limulus polyphemus*). The blood from small-young (2.0–3.0 grams) animals was obtained by heart puncture, pooled, and the supernatant serum removed for future study. Aliquots of sera of large-old crabs (3.2–4.5 kg.) were obtained in the same manner. The sera were dialyzed against buffer (pH 8.6) and analyzed by starch-gel electrophoresis. Such dialyzed samples gave considerably better resolution than comparable non-dialyzed samples. Since spectrophotometric measurements (490 λ) demonstrated more haemocyanin in the serum of the older animals, the serum from large crabs was diluted (with buffer) until it approximated that of small crabs.

Electrophoresis of the sera from both age groups was carried out by running the two samples in the same gel. The small Limulus serum revealed 7 distinct bands, all migrating towards the anode area, whereas the serum from large crabs revealed only 4 distinct bands and a fifth slower migrating diffused band. The 4 fastest migrating bands in the large Limulus serum were comparable in mobility and stainability with that of the 4 fast bands of the small Limulus serum. The remaining 3 fractions present in small Limulus serum migrated considerably faster than the fifth diffused band of large Limulus serum and thus were not comparable.

Preliminary evidence indicated that a blood protein, migrating like a gamma globulin towards the cathode area, was present in whole blood aliquots and sometimes in sera of young crabs. To date, this gamma globulin-like fraction has not been found in the sera of older animals. Since gamma globulins have not previously been found in the blood of invertebrates, this observation is under further study.

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The effect of volume on the termination of pregnancy. A. I. Csapo and M. A. Lloyd-Jacob.

Earlier experiments indicated that an increase in uterine volume facilitates parturition and that this effect of volume on delivery can be balanced by progesterone therapy. In the present experiments we kept the progesterone treatment constant and altered uterine volume so as to demonstrate conclusively that an increase in uterine volume hastens delivery.

Twenty-five days pregnant rabbits were ovariectomized and their placentae dislocated. Endogenous progesterone was substituted by a 6 days' decremental progesterone treatment ranging from 2.0 mgm. to 0.5 mgm./day. Such animals with a total uterine volume of about 200 ml. began to deliver 24–48 hours after surgery at the 1.5–1.25 mgm./day progesterone level. Delivery was prolonged for several days and was often only partial. However, if the content of one uterine horn was transferred into the other, delivery usually began within twelve hours after operation and the excess volume was quickly delivered.

When the uterine volume was controlled by dummy babies made of a mixture of paraffin and wax it was the horn carrying a larger volume which delivered first. Delivery from a horn with exceedingly small volume often failed altogether. All these observations point to the same conclusion; namely, that volume and progesterone have opposing effects on the parturient activity of the uterus. When the ovaries were removed in a group of animals subjected to decremental progesterone therapy and the content of only one horn was replaced by dummy babies, the dummies were first delivered in spite of the excess volume of the intact horn due to fetal growth. This finding is in good agreement with earlier observations that functional placentae exert a local effect on the myometrium, and also with the conclusion that excess progesterone can balance the effect of increasing uterine volume on facilitating delivery.

Ionic relations in the Fundulus egg. Philip B. Dunham and David C. Shephard.

Jacques Loeb's experiments on heart beat cessation in intact *Fundulus* embryos immersed in KCl solutions have been repeated. His observation that the heart beat fails to recover as long as the embryo is left in distilled water, but resumes after being placed in sea water, was confirmed. His suggestion that the excess K cannot leave the egg unless there are positive ions outside for exchange was checked by following the net changes in the Na and K contents of 8–12-day *Fundulus heteroclitus* embryos in different solutions. When eggs are placed in 0.5 *M* KCl, the K content increases and the Na content decreases. Net changes are effectively complete in 30 minutes. When returned to sea water, the K and Na contents return to their original levels in a similar period of time. Fluxes of Na^{24} showed rapid equilibration of most of the Na content of the eggs. However, the K content of eggs in sea water is very slowly exchangeable with K^{42} (25% in 75 hours). When KCl-poisoned embryos were placed in distilled water, the K content decreased rapidly to its normal level, but the heart beat did not recover unless the eggs were subsequently returned to sea water. K^{42} efflux from KCl-poisoned eggs is rapid and of the same rate in sea water and distilled water. The Na content of embryos moved from sea water to distilled water was significantly higher than that of the KCl-poisoned embryos, although lower than in embryos in sea water. Thus, the failure of the heart beat to resume in distilled water is clearly not due to the retention of the initial high K content as suggested by Loeb, but is probably due to an imbalance in the Na:K ratio in the blood of the embryo. This hypothesis was tested by experiments on exposed hearts of dechorionated embryos. The heart beat stopped in 5 seconds when exposed to solutions with Na:K ratios of 5:1 or lower, but recovered quickly if the ratio was increased to 10:1. These results were independent of total concentration over at least a two-fold range of concentrations.

Studies of melanin biosynthesis in the ink sac of the squid (Loligo pealii). Thomas B. Fitzpatrick, Makoto Seiji, Robert Simpson and George Szabo.

Melanin particles contained in the cells lining the ink sac and in the ink "milked" from the ink sac have been isolated by differential centrifugation method, followed by density-

gradient centrifugation. Tyrosinase activity was studied in the isolated particles. In mammals, the *melanosome* is a varying-sized cell organelle with a distinctive internal structure and tyrosinase activity. The *melanin granule* represents a later stage of development of melanosome; the melanosome becomes progressively melanized and the tyrosinase activity disappears, presumably because it is masked by the deposition of the melanin polymer on the particle. The melanin granule is a heavily pigmented particle without detectable tyrosinase activity. This sequence of events from an enzymatically active particle to an inert dense particle also appears to occur in the squid ink sac. Melanin particles isolated from homogenates of the ink sac have marked tyrosinase activity, while those in the ink have no tyrosinase activity. It was possible to solubilize the tyrosinase from the melanin particles in the ink; thus the ink particles contained a masked tyrosinase. In contrast to mammalian pigmented tissues, however, the ink sac has been found to contain most of the tyrosinase activity in the soluble fraction. Characterization of the ink sac tyrosinase showed it to be similar to mammalian tyrosinase: tyrosine and DOPA were the most active substrates, copper-binding agents inhibited enzyme activity, and the same type of lag in the oxidation of tyrosine occurred.

Although the pigment in the eye and in the "chromatophores" of the skin of the squid occurs in granular form, similar in size and density to melanin granules, these do not contain measurable tyrosinase. The pigment in the granules has the solubility and other properties of ommochromes which have previously been demonstrated in squid skin and eye.

Influence of tail skin, epidermis, and dermis on forelimb regeneration in the axolotl. RICHARD W. GLADE AND NANCY J. SCOTT.

Tail tissues were transplanted between black and white axolotls (body lengths 7 to 11 cm.) to 43 fore- or upper arm stumps. Whole mock grafts and portions of actual grafts were analyzed for percentages of epidermal, glandular, connective tissue, and muscle nuclei. Tail skin, epidermis, and dermis were transplanted singly or in combination with spinal cord from the tail. Contralateral limbs, amputated at the same level as the experimentals, served as controls. Epidermis and dermis were isolated by allowing pieces of skin to remain in 100% Holtfreter's solution 1–2 hours at room temperature. The tissues could then be peeled apart, and proved to be viable. Dermis-free epidermis was also obtained by removing the wound epithelium from denuded sections of tails.

The cartilage structure of 22 useful experimental regenerates has been studied to date and correlated with percentages of component cells in the grafts. When epidermal cells constituted 90–95% of the graft (isolated epidermis), the regenerates were either normal, in one out of five cases (⅕ cases), or slightly inhibited (3 digits, ⅘ cases). When connective tissue cells formed 22–28% (whole skin) or 88–92% (isolated dermis) of the graft, more severely inhibited regenerates appeared. Moreover, in $^{10}/_{17}$ of these cases, cartilages developed which did not appear to be homologous to those of the limb. In $^{5}/_{10}$ cases, these were of the axial type.

The frequency of various degrees of limb inhibition and the development of non-limb or axial cartilages could not be correlated with the implantation of spinal cord. The same lack of correlation applied to the presence of tail muscle fibers which adhered as a contamination (maximum 0.2% of the nuclei of the graft) to some of the grafts.

It is concluded that tail skin may express its own regionality in the cartilage pattern of the forelimb regenerate. The factors responsible probably reside in the dermis.

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Strontium utilization by Arbacia punctulata. I. P. GOLDRING AND H. I. HIRSHFIELD.

An artificial sea water containing strontium as a substitute for calcium was used to test its effect upon the activation, fertilization, and subsequent development of *Arbacia*. The medium was free of phosphate and sulfate, due to their low solubility with strontium in water. The bulk of the anions were in the form of chlorides with a small quantity of bicarbonate present.

A control solution (an artificial sea water containing equivalent amounts of calcium and the same components as the strontium medium) was used. Parallel studies using calcium-free artificial sea water and filtered sea water were carried out.

The visible effects of the artificial media, whether it was calcium or strontium, indicated that anion deficiencies existed, probably sulfate and phosphate. The purity of the salts used was critical. The results with the calcium and strontium artificial media were surprisingly similar. Insemination of strontium-exposed eggs with sperm in strontium sea water resulted in no activation or fertilization. Normal sperm, however, were capable of activating a high percentage of eggs in strontium medium. Actual cleavage numbers were lower than in normal eggs and many abnormal forms were present. A higher degree of mortality and asynchrony in development were noted. Neither medium could support development past the prism stage. Aliquots containing different stages of development, up to early gastrulae, from either medium transferred into normal filtered sea water produced normal plutei. Mixtures of the artificial strontium and calcium media could not produce normal long-armed larvae. Developmental arrest at the prism stage in the artificial media or their combinations could be maintained for at least 5 days.

Skeletal spicules were formed in both types of artificial media, but their composition is unknown.

Comparisons of the above studies with similar studies of *Chaetopterus* indicate that there is a greater need for skeletal metabolites in *Arbacia* than in *Chaetopterus*. Although the mortality rate was higher, trochophores were produced which were mostly abnormal.

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An accessory small nerve fiber in a mechano-receptor. K. GOTO AND W. R. LOEWENSTEIN.

Besides the well-known myelinated axon (large fiber), a fine non-myelinated axon (small fiber) enters the Pacinian corpuscle. The large fiber is clearly the afferent axon of the sense organ: it transmits the impulses elicited by mechanical stimulation of the receptor. It conducts at 25 m./sec. (32° C.). The small fiber is not excited by mechanical, thermal, or chemical stimulation of the corpuscle. It conducts at 0.65 m./sec. Under certain conditions, we have found it to have a facilitatory action on the receptor process. Single mesenteric Pacinian corpuscles were isolated together with a length of its extra-corpusclar nerve supply containing the large and the small fiber. When the small fiber was stimulated selectively outside the corpuscle so that only the small fiber impulses (but not the antidromic fast fiber impulses) run into the corpuscle, the generator potential of the nerve ending of the fast fiber in response to a given mechanical stimulus was reversibly enhanced by about 10%, and the mechanical threshold for firing of impulses in the fast fiber lowered by about the same amount. However, the effect could be shown only in 12 out of 91 preparations; and even in the rare successful cases, it lasted only a few minutes. The physiological role of the small fiber is thus as yet unclear. Because of its insensitivity to various modalities of sensory stimuli, it is likely to be an efferent axon. But whether receptor facilitation is its function, as in the case of the sympathetic small fiber system of certain cutaneous touch receptors (Loewenstein, J. Physiol., **132**: 40, 1956), or whether its role is merely to control the tonus of the small blood vessel inside the corpuscle, is quite uncertain.

A densitometric technique for measuring the rate of reaggregation of dissociated sponge cells. BERNICE GRAFSTEIN.

Classical studies of reaggregation of sponge cells, based on the number and size of aggregates formed after a finite period of time, provide only an indirect and qualitative estimate of the rate at which aggregation proceeds. With the present method, the aggregation rate was determined quantitatively by measuring the rate of disappearance of unaggregated material.

Dissociated cells were prepared by squeezing 10 g. of sponge through #14 standard bolting cloth into 50 ml. calcium- and magnesium-free sea water at 0° C. Suspensions made from either *Microciona prolifera* or *Haliclona permollis* contained between 12×10^4 and 20×10^4 cells per mm.^{3} Aggregation was initiated by adding 1 ml. cell suspension to 4 ml. filtered sea water in a petri dish. After an interval of between 2 and 60 minutes, the aggregated material was removed and aggregation arrested by pouring the 5-ml. sample through three layers of

bolting cloth into a cold test tube containing 25 mg. crystalline sodium citrate. The "unaggregated" material remaining in suspension consisted of dissociated cells and aggregates no larger than 15 μ in diameter. The optical density of this material was read on a Coleman Junior Spectrophotometer at 660 or 570 mμ. Readings were converted to cell numbers by means of appropriate calibration curves.

There was found to be a marked difference between the rates of reaggregation of *Microciona* and *Haliclona* at 14.5° C. With *Haliclona*, there was a logarithmic decrease in the amount of unaggregated material, with a half-time of 45 minutes. With *Microciona*, there were two phases of logarithmic decrease. In the initial 15 minutes, the half-time was 10 minutes; subsequently it was about 220 minutes. This difference may be important in the process of separation of cell types that occurs in the aggregation of a mixture of cells from different sponges.

Performed during the tenure of a Grass Foundation Fellowship.

Effect of aconitine on the giant axon of the squid (Loligo pealii). WALTER H. HERZOG.

Aconitine is known to cause repetitive responses in nerve after a number of short shocks. The following observations were made with either internal double wire stimulating and recording electrodes or internal KCl-filled glass electrodes and external stimulation. In axons with action potentials greater than 100 mV. or resting potentials greater than 53 mV. there was no oscillation following action potentials produced by short square wave shocks. Repetitive firing (maximum of five impulses) occurred in most axons with suprathreshold shocks of 15 to 20 milliseconds duration.

Without stimulation aconitine (1×10^{-6} g./cc.) had no effect on resting potential or on size and shape of action potential. However, if the axon was stimulated at a regular rate, resting potential decreased gradually by five millivolts and, concomitantly, damped oscillations appeared which increased in size until repetitive firing occurred. The repetitive firing usually continued for 20 to 70 seconds. Higher stimulation rates and/or longer time was necessary to elicit repetitive responses with lower concentrations (1×10^{-7} and 3×10^{-7} g./cc.). At the end of the repetitive burst the axon was depolarized by ten to fifteen millivolts. Repolarization occurred in 10 to 15 minutes. Repolarization occurred in less than three minutes if the axon was placed in sodium-free solution (sodium replaced by choline). Aconitine (1×10^{-4} g./cc.) produced a depolarizing block without stimulation.

Measurements were made of current-voltage relation in these axons. Without stimulation aconitine produced no change in current-voltage curves. With stimulation at a rate producing oscillations the current-voltage curve indicated a decreased membrane resistance. Decreasing external sodium from 423 to 168 mM./L. decreased the height of the action potential and oscillations, and increased the membrane resistance to control levels.

This evidence indicates that the action of aconitine in squid axons is due at least in part to increased sodium conductance.

Nucleotide metabolism in developing Arbacia. HENRY I. HIRSHFIELD, SELMA B. ZIMMERMAN AND ARTHUR M. ZIMMERMAN.

Eggs of *Arbacia punctulata* were incubated in 0.5 μC./ml. thymidine-H^3 sea water for one hour, or five minutes prior to insemination. They were maintained in this medium until the metaphase of the first mitotic cleavage and then placed in Bouin's fixative. Subsequently they were embedded and sectioned at 5 μ. The prepared slides, stripped with AR. 10 autoradiographic film, were incubated 25 days prior to development of the film. Similar studies were made using 1.12 μC./ml. uridine-H^3. Uptake of the thymidine and its incorporation into chromosomal DNA was confirmed by the use of DNA-ase. No specific localization or incorporation of the uridine was found.

The duration of incubation with thymidine was not significant. Incorporation of thymidine into the chromosomes in those eggs kept one hour prior to fertilization and those eggs kept five minutes prior to fertilization was about the same. This suggests that active uptake and utilization probably occurred during or after fertilization.

The analogous utilization of cytidine in DNA synthesis was investigated by immersion in cytidine-H^3 1.6 μC./ml. for one hour prior to fertilization and five minutes after fertilization. Surprisingly, no comparable localization of cytidine to thymidine was found within the nucleus. The autoradiograms of cytidine were similar to those of uridine-H^3.

The lack of incorporation of cytidine and uridine cannot be easily explained. One possible interpretation is that uridine and cytidine or precursors are readily available in early cleavage stages. Thymidine may be present in extremely low concentrations and produced as needed from other nucleotides or precursors. Its rapid uptake and incorporation in the chromosomes indicate that, when available, thymidine will be incorporated into DNA, possibly sparing conversion of other nucleotides or thymidine precursors.

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Effect of electrical currents on the initiation of nerve impulses in a Lorenzinian ampulla. NOBUSADA ISHIKO AND WERNER R. LOEWENSTEIN.

When the internal pressure of Lorenzinian ampullae (*Mustelus canis*) is raised above the external pressure, a generator potential is produced in the receptor elements. The generator potential and the frequency of the resulting nerve impulses increase with the pressure differential. The impulse frequency at any given pressure differential is markedly altered by the flow of electrical currents through the ampulla. Current flow in the direction of ampulla swelling to pore enhances, and, in the reverse direction, depresses the frequency. The minimal effective current is 5×10^{-9} amps. (Current was passed with a micro-electrode placed on the swelling and a silver-silver chloride wire placed at the pore opening.) The effect is not due to a direct excitation of the nerve ending or fiber of the receptor, but requires the presence of a generator potential produced by pressure: when the pressure-generator mechanism is turned off by setting the pressure differential near zero, currents as high as 10^{-5} amps. are ineffective in eliciting generator or action potentials (the nerve fiber was normally excitable by directly applied electrical stimuli). The minimal current required to flow through the ampulla in order to produce a given enhancement of background impulse frequency (minimal current) is inversely related to pressure or generator potential. For example, the minimal current at 60 cm. (Hg) pressure is about one hundredth of that at 10 cm. (Hg). (This pressure change corresponds to an approximately four-fold change in generator potential and a five-fold change in background frequency.) At constant pressure, the enhancement or depression of frequency increases roughly linearly with the logarithm of current.

Effect of x- and gamma radiation on the growth rate and conidia formation in Neurospora crassa. JOHN KEOSIAN.

Unless otherwise specified, radiation was from a 182 Kv. machine, inherent filtration of 0.15 mm. Cu, dose rate 4860 r per minute, total dose 9000 r.

1. A small but consistent post-irradiation increase in growth rate was previously reported in *Neurospora* cultured in growth tubes. Present studies using longer tubes, showed the increased growth rate to persist to the end—a period of more than four days.

2. Irradiation of the growing frontier of the mycelium causes the early appearance of a heavy band of conidia at the frontier. The effect of radiation was tried on an albino, aconidial, slow surface-growing strain of *Neurospora*. No conidia appeared, but the irradiated cultures, after a short lag, changed to a faster, heavier, aerial growth. The same change occurred in the controls two days later.

3. In a deficient medium the wild type shows a slow, sparse, surface growth which does not develop conidia. Irradiation of the frontier of these cultures caused the appearance of a sparse band of albino conidia. Four successive irradiations of these tubes, permitting about two inches of growth after each exposure, produced four successive bands of albino conidia in each tube.

4. The R.B.E. (relative biological effectiveness) of gamma radiation (Cs-137 irradiator) was tested by exposing *Neurospora* cultures in Falcon plastic petri dishes to doses ranging

from 1000 to 100,000 r. No frontier conidia formed. X-radiation using plastic dishes also gave negative results. X-radiation produces frontier conidiation in *Neurospora* grown in Pyrex dishes. The role of Pyrex vs. plastic on this effect is under investigation.

Krebs and pentose cycle dehydrogenase systems in eggs of Spisula as measured with a tetrazolium salt. EVELYN KIVY-ROSENBERG AND FRANCES RAY.

The quantitative study of substrate-dependent dehydrogenase activity was continued with the investigation of uninseminated and inseminated eggs of the bivalve *Spisula*. The series of substrates utilized included those which require no cofactor as well as those with DPN or TPN as cofactors. Among the substrates were four involved in the Krebs cycle (isocitrate, using TPN as cofactor; alpha-ketoglutarate, and malate using DPN; succinate with no cofactor) and two in the pentose cycle (glucose-6-phosphate and 6-phosphogluconate, both requiring TPN). As has previously been reported, the tetrazolium salt utilized as hydrogen acceptor was 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT).

Since the number of eggs obtainable from a single clam would not suffice for a complete assay of an uninseminated and inseminated set, the eggs from several females were pooled for each assay. Homogenates of each of several pools of uninseminated and inseminated eggs were incubated aerobically for one hour at 37.5° C. in media containing INT and substrate or appropriate controls. Formazan (reduced tetrazolium) was extracted and the quantity measured spectrophotometrically. The substrate-dependent dehydrogenase activity was expressed as micrograms of formazan per milligram of protein.

Of the seventeen substrate-dependent dehydrogenase systems assayed, two of the Krebs cycle were most active: namely malate which ranked first and isocitrate, second. Succinate was in about position 7 and alpha-ketoglutarate was at 6. However, the latter was the least consistent of the group in that its activity was positive in most cases but not in every case tested. Thus succinic dehydrogenase activity, though lowest of this cycle, did not rank as low as was seen earlier (Biol. Bull. **119**, 1960) for *Asterias*. With respect to the pentose cycle, the rank of activity of the glucose-6-phosphate-dependent dehydrogenase system and that of 6-phosphogluconate were fourth and fifth, respectively.

The relative activity was in general similar for inseminated as for uninseminated eggs.

Hormonal activity of sinus glands in Carcinus maenas from different geographic localities. L. H. KLEINHOLZ.

Differences in distal retinal pigment hormone (DRPH) activity had been observed when sinus gland extracts of *C. maenas* were tested at several marine laboratories. To insure that such differences are not attributable to the species used as test animal in each locality, standardized preparations and testing methods were necessary. Sinus glands, eyestalks from which the sinus glands had been removed, and whole eyestalks of *C. maenas* from Woods Hole (WH), Plymouth (P), Roscoff (R) and Naples (N) were dried for 5 hours at 105° C. Sets of the three tissues were collected within two weeks. These, as well as similar tissues prepared at Kristineberg (K), were subsequently tested for activity on dark-adapted *Palaemon adspersus*. Extracts of sinus glands, in a concentration of 10 per 1.0 ml. of water, when injected into dark-adapted test animals, produced the following retinal response indices: WH, 0.086; R, 0.087; N, 0.098; P, 0.171; K, 0.170; retinal indices for uninjected dark-adapted control animals averaged 0.029. Similarly injected extracts of eyestalks from which the sinus glands had been removed effected the followed indices: WH, 0.165; P, 0.162; R, 0.173; N, 0.174; K, 0.174. Extracts of entire eyestalks gave these indices: WH, 0.178; R, 0.171; N, 0.174; K, 0.184. The observed results indicate that sinus gland extracts from *C. maenas* of three geographic regions, even though injected in relatively high concentrations, contain little light-adapting DRPH; similar extracts from *C. maenas* of Plymouth and of Kristineberg, however, produce nearly maximum responses. It is also evident, from the light-adapted responses produced in the test animal by extracts of sinus glandless eyestalks, that light-adapting DRPH is present in tissues of the eyestalks other than the sinus gland.

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Enzymatic inactivation of crustacean distal retinal pigment hormone. L. H. KLEINHOLZ AND FRANCES KIMBALL.

An enzymatic mechanism has been indicated as inactivator of pigmentary effector hormones from the crustacean eyestalk; such inactivation of the light-adapting distal retinal pigment hormone (DRPH) was examined. Standardized assay determinations show that aqueous extracts of eyestalks of *Palaemonetes* (20 eyestalks per 1.0 ml.) produce average DRP indices of 0.20 when injected into dark-adapted test *Palaemonetes*, compared with average indices of 0.05 in uninjected dark-adapted controls. Progressive decline in DRPH activity occurs in aliquots taken at intervals from eyestalk extracts kept at 25° C.; at this temperature, DRPH activity may be completely absent after 12 to 18 hours. On the other hand, DRPH activity remains essentially unaltered over a 24-hour period if a freshly-prepared eyestalk extract is heated at 100° C. for two minutes. Addition of antibiotics to unheated eyestalk extract did not prevent marked decrease in hormonal activity. The pH optimum for enzymatic inactivation of DRPH was examined in appropriately buffered mixtures of eyestalk extract, from which DRPH had been removed by dialysis, and of partially-purified hormone as substrate. Buffered experimental and control mixtures were incubated for 6 hours at 38° C.; control mixtures contained enzyme extract denatured by heating for two minutes at 100° C. The pH of each mixture was verified at the end of each incubation period. DRPH content was assayed by determining DRP indices in dark-adapted *Palaemonetes* after injection of the buffered preparations. Enzymatic inactivations of DRPH at pH 5.1, 6.0, 6.7, 7.3, 8.0 and 9.0 are, respectively, 6%, 15%, 43%, 65%, 53% and 5%. Optimum for the inactivating enzyme is about pH 7.5. The enzyme occurs also in crustacean nerve cord and in hypodermis; its specificity is being examined in further studies.

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Anodal inhibition of the regeneration of Tubularia crocea within an electric current. STEPHEN LEVIN.

With an apparatus similar to that described by Barth (1934), constant currents were passed through 10-mm. regenerating stem sections of *Tubularia crocea* oriented parallel to the direction of ion flow. Fresh sea water was constantly circulated through the current chamber at an exchange rate of approximately 450 ml./minute. The stems were left in a continuous curent density of 80 δ ($\delta = \mu$amperes/mm.^{2}, cross-sectional area of sea water carrying current) for 24 hours, at the end of which time they were removed and placed in a chamber supplied with circulating sea water for two days. The polarity of regeneration was then observed.

Of the stems oriented with their distal ends closest to the anode, 18% regenerated hydranths at the original distal end only; 25% exhibited reversal of polarity, with a hydranth regenerated at the proximal end only; 48% of the stems regenerated hydranths at both ends, and 9% failed to regenerate at all.

Seventy-two per cent of the stems oriented with their proximal ends closest to the anode regenerated hydranths at the distal end only; 1.5% of the stems regenerated at the proximal end only (*i.e.*, reversals); 3.5% regenerated hydranths at both ends; 23% failed to regenerate at either end.

Barth (1934) has reported a 7.3% frequency of polarity reversals for stems oriented toward the anode, using a current density of 62.5 δ. The higher frequency of reversals obtained in our laboratory (25%) may have been the result of the higher δ value.

An attempt was made to determine the period of critical effectiveness of the current. Batches of stems were placed in the current for the first 12 hours after cutting, and others for the second 12 hours of regeneration. After removal from the current, the stems were allowed to regenerate for three days in a water chamber. No significant difference could be found among the data obtained from the two sets. This may have been due to the wide variability of rate of regeneration in the stems in a single batch, since the current thus acts on different stages in the regeneration process of different stems.

Experimental missed abortion and missed labor. M. A. Lloyd-Jacob and C. B. Kovacsics.

Csapo's theory that these functional disorders of the pregnant uterus are due to an imbalance of three major controlling parameters, namely estrogen, progesterone, and volume, was tested experimentally. Missed abortion and labor were produced predictably by the method of Csapo and Lloyd-Jacob, who maintained pregnancy with progesterone in rabbits whose ovarian and placental endocrine functions had been suspended by surgery. Delivery of the dead litter during progesterone treatment was determined by the dose administered and by the uterine volume (at the day of surgery). Increased progesterone balanced the facilitating effect of volume on the evacuation of the uterus.

The ovariectomized and placental dislocated animal not only failed to complete delivery *during* prolonged progesterone treatment but also *after* such treatment in case of a small uterine volume. However, the animal did deliver during progesterone treatment if it was inadequate (too low, or of short duration), or after the discontinuation of effective progesterone treatment if it was combined with or followed by estrogen therapy.

These observations, together with the findings of Takeda and Csapo on the electrophysiological properties of the myometrium under similar experimental conditions, suggest that the delivery of a dead litter is facilitated by the withdrawal of progesterone, by an increase in uterine volume, and by estrogen therapy. These joint studies predict that missed abortion and labor in women occurs when placental progesterone production continues after fetal death *in utero*. Since the uterine volume is static or is decreasing after fetal death and placental estrogen production greatly diminishes, little progesterone maintains pregnancy. The estrogen support of the myometrium being insufficient, uterine activity is inadequate when progesterone withdrawal permits the evacuation of the uterus. The experiments described suggest a rational therapy.

Oviparity versus ovoviviparity in the brine shrimp, Artemia. J. H. Lochhead.

The ovisac of *Artemia* is surrounded by unicellular shell-glands. Sometimes these glands discharge a copious brown secretion and the animal then lays hard-shelled eggs which may not hatch for several years. Sometimes the shell-gland secretion is scanty and forms only a thin membrane around the eggs. These then develop rapidly to naupliar larvae which emerge from the parent after hatching in the ovisac. Females release either eggs or larvae about once every four days. Which type of reproduction will occur presumably depends on external factors, but the literature dealing with such factors has been conflicting.

Experiments were set up in which animals of known age were subjected to different salinities, oxygen tensions, and foods. In some cases individual animals were observed daily over a period of several weeks. Where possible water was changed daily, to minimize differences which might develop between control and experimental cultures. None of the factors tested was found to exert an absolute control. Oviparity was most frequent in animals fed on algae or on liver. Ovoviviparity was most frequent in animals fed on yeast or kept at a high salinity (about six times that of sea water). However, both types of reproduction occurred under all the conditions tested and in animals both with and without hemoglobin in the blood. Individual animals sometimes changed, in either direction, from one type of reproduction to the other. An animal having once changed, tended to maintain the new type of reproduction in successive instars, although other animals under identical conditions continued reproducing by the other method. Age of the individuals did not appear to be a critical factor, and both types of reproduction were observed even in primiparous animals.

The lamprey eel blood clot. L. Lorand.

The blood plasma of higher vertebrates (*e.g.* human and bovine) contains a globulin, called fibrin stabilizing factor (FSF), whose apparent physiological function is to cement together the fibrin units of a blood clot (Physiol. Rev., **30**, 742, 1954). A clot consisting of fibrin alone is mechanically weaker than the natural plasma clot and, unlike the latter, it can be dispersed by a number of solvents such as 30% urea or 1% monochloroacetic acid. This difference in solubilities between the two types of clot allows for easy recognition of the func-

tioning of FSF in blood. Similarly to fibrinogen, FSF appears to be activated by thrombin and full potency is preserved only in the presence of sulfhydryl compounds. Pathologically, there may be a relative (Proc. Soc. Exptl. Biol. Med., **89**, 45, 1955) or total (Thromb. et Diath. Haemorrhagica, **5**, 179, 1960) lack of FSF in plasma and, in spite of normal clotting and bleeding times, the latter results in grossly impaired wound healing. Since FSF seems to be as important a component of blood plasma as fibrinogen itself, it was of interest to ask where in the phylogenetic scale FSF makes its appearance. This paper reports that stored citrated plasma of *Petromyzon marinus*, when clotted by recalcification in the presence of 25 m*M* cysteine and homologous skin thromboplastin (Doolittle), developed clots which were insoluble in 30–40% urea or 1% monochloroacetic acid. Further, similarly to bovine fraction I (Armour), ethanolic fraction I (Cohn) of lamprey plasma is contaminated with FSF. Such lamprey fibrinogen preparations, after clotting with bovine thrombin in the presence of 25 m*M*. cysteine and calcium chloride, give rise to urea- and monochloroacetic acid-insoluble clots.

This work was aided by NIH grant H–2212. Thanks are due to Warner Love for providing the plasma and to Russell Doolittle for advice regarding lamprey blood.

The effect of cobalt, ribonucleic acid, and ribonuclease on the development of Arbacia punctulata. G. M. Mateyko.

It has been reported that cobalt may bring about persistent nucleoli during mitosis. Moreover, it has an inhibiting action on ribonuclease and reacts with sulfhydryl groups. Accordingly, the development of fertilized eggs of *Arbacia punctulata* was followed in sea water containing varying concentrations and combinations of cobalt chloride, ribonucleic acid, and ribonuclease, and the patterns of cleavage through the pluteus stage were studied.

At concentrations below 0.01 mg./ml. for $CoCl_2$, 5 μg./ml. for RNA, and 0.01 mg./ml. for RNA-ase, no morphologically detectable developmental retardation was evident, nor was there any acceleration. Above these levels growth was retarded. A specific response to cobalt, elicited in addition to the depression in the rate of cleavage, was the distortion of the prism and young pluteus to a form simulating a pollen grain. In 0.02 mg./ml. cobalt, deformed prisms occurred in 48 hours, and at 0.04 mg./ml., bloated prisms with swollen arms were characteristic. In 0.06 mg./ml. cobalt, the retardation and bloating to the *pollen grain* stage is characteristically reached by 36 hours. Internal organization is lost and isolated cells fill the sac-like arms and body in 96 hours. Although skeletal development proceeded in 0.04 mg./ml. cobalt and higher, the spicules were deformed and incomplete. Separation and dispersion of blastomeres was striking in cobalt (0.08 mg./ml.) especially after 8 hours, in that morulae hatched out of the fertilization membrane, and subsequently the blastomeres disaggregated.

Neither RNA-ase nor RNA in physiological amounts, nor in higher concentrations (0.1 mg./ml. and 50 μg./ml., respectively), minimized or reversed the cobalt effect. In fact, each, especially ribonuclease, depressed still further the rate of cleavage.

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Digestion of the Fundulus chorion by the hatching enzyme. Roger Milkman, Barry F. Wood and Delbert E. Philpott.

The *Fundulus heteroclitus* egg chorion has previously been shown to consist of two layers. The thick inner layer is tough and digestible by the hatching enzyme, while the thin outer layer is weak but resistant to the enzyme.

The changes in the chorion during hatching have been studied. Electron micrographs of cross-sections of intact chorion show a uniform membrane about 6–8 microns thick, within which are suggestions of 7–8 roughly defined layers. There is a thin outer layer present (1/6 to 1/3 micron thick), which remains undigested by the hatching enzyme. Three progressive changes are apparent in cross-sections of chorions removed at successive times during hatching. First, the inner edge becomes rough and fibrous. Second, the digestible layer's density is reduced. Third, holes appear in this layer. In some places the layer is completely digested away. The roughened edge is seen after two minutes' exposure to the enzyme; the holes

begin to appear after four minutes; after about seven minutes, the process is complete. The weak, indigestible outer layer is all that remains, and the fish breaks out of it with a lash of its tail.

Experiments have shown that enzyme from recently hatched eggs will attack the chorions of intact eggs wherever the protective layer has been filed away. If this filing is done in a ring around the egg, the chorion comes apart into two hemispheres and can easily be removed, leaving the embryo alive and intact.

This work supported by a grant from NIH (Milkman) and an NSF Cooperative Summer Fellowship (Wood).

Oxygen uptake in short pieces of Tubularia stems. JAMES A. MILLER, JR., LORALEE L. PHILPOTT AND C. LLOYD CLAFF.

Reconstitution in *Tubularia* is initiated by the presence of a cut surface. However, if two cut surfaces are close to each other (*i.e.*, in stems 1½ mm. or less in length), reconstitution may fail completely and if it does take place proceeds slowly and produces very small hydranths. These results suggest that influences which emanate from the cut surfaces inhibit reconstitution. Since the perisarc is relatively impermeable and metabolic exchange is chiefly through the cut ends, the influence of the cut ends on oxygen uptake of short pieces was determined. Measurements were made with Cartesian divers on stems at 18° C. which had been treated with a solution of streptomycin in filtered sea water. Using the oxygen uptake of 3-mm. stems as a standard of reference it was found that doubling the surface area (two 1½-mm. pieces) or tripling the surface area (three 1-mm. pieces) without altering the mass of tissue failed to produce comparable increases in oxygen uptake. Comparisons between the oxygen uptake of one 1-mm. piece and three 1-mm. pieces in 2 μl. showed that the single 1-mm. piece consumed over one-half that of three 1-mm. pieces in the same volume of sea water. Since in all these experiments CO_2 was absorbed by the NaOH in the diver it is concluded that some metabolic depressant other than CO_2 is liberated from the cut surface. Although it is tempting to correlate the observed metabolic depression and inhibition of reconstitution in short pieces, as yet a cause-effect relationship between these two phenomena remains to be demonstrated.

Malic dehydrogenase isozymes of Asterias forbesi and Arbacia punctulata ovary. RICHARD O. MOORE AND CLAUDE A. VILLEE.

The relative reaction rates of certain dehydrogenases with several pyridine nucleotide analogues have been implicated in the discernment of evolutionary relationships. In 1960 Villee reported that the malic dehydrogenases of comparable tissues, *e.g.* ovary, of related marine animals were more similar in their DPN-analogue activities than those of different tissues, *e.g.* ovary, gut, muscle, from the same species. To determine whether a single enzyme was present in each tissue with varying properties among tissues, or rather that each tissue contained several malic dehydrogenases with different pyridine nucleotide specificities so that the observed species and tissue patterns could be accounted for on the basis of varying concentrations of several malic dehydrogenases, an attempt has been made to demonstrate the presence (or absence) of multiple molecular forms of this enzyme in the ovary of *Asterias* and *Arbacia*. The tissue was homogenized, centrifuged and the proteins of the supernatant fluid fractionally precipitated with $(NH_4)_2SO_4$. The fraction with highest malic dehydrogenase activity was dialyzed and placed on a DEAE-cellulose column. The malic dehydrogenase did not adsorb to the DEAE-cellulose. The DEAE-cellulose elulate was placed on a carboxymethyl-cellulose column and 1.3- or 2.6-ml. fractions collected over a gradient of 100 ml. tris-succinate-mg-acetate buffer, pH 5.4, and 150 ml. 1.0 *M* KH_2PO_4. Malic dehydrogenase activity with DPN, 3-acetylpyridine-DPN (APDPN), deamino-DPN, thionicotinamide-DPN, and 3-pyridine-aldehyde-DPN was followed spectrophotometrically at critical points throughout the procedure and on individual fractions eluted from the carboxymethyl-cellulose column. Supernatant fractions of the original homogenates were also subjected to zone electrophoresis on starch. The data from these experiments indicate that three molecularly distinct malic dehydrogenases exist in ovaries of *Asterias* and *Arbacia*, each with a different DPN-analogue pattern. The further

observation has been made that the enzyme with major APDPN activity decreases during the breeding season from early June to August, and that this same enzyme is more labile to laboratory manipulation (*e.g.* frozen storage).

Studies on the isolated islet tissue of the toadfish (Opsanus tau): glucose-6-phosphate and 6-phosphogluconate dehydrogenases. JOSEPH F. MORAN, JR.

As part of a systematic study of the isolated islet of the toadfish, and because of the known effect of glucose on the release of insulin from the beta cell, we have been investigating the enzymes involved in the metabolism of glucose by islet tissue. In the present study the glucose-6-phosphate (G-6-PD) and 6-phosphogluconate (6-PGD) dehydrogenase contents of toadfish tissues were determined by measuring the rate of reduction of triphosphopyridine nucleotide (TPN) at 340 mμ in a Beckman spectrophotometer under standardized conditions in the presence of cyanide and magnesium. Weighed samples of islet (1–4 mg.) and other tissues were homogenized in glycylglycine buffer and aliquots (5–20 μl) were added to the assay system; the final volume was 300 μl. The enzyme activity was expressed as the change in the optical density per minute per mg. wet weight of tissue added.

The average G-6-PD content of the islet tissue of 10 animals was 0.023; this value is 33–50% of that found in liver, ovary, testis, gill and brain, and 8–15% of that found in the heart and kidney. The 6-PGD content of islet (.023) was the same as that found in the other tissues, except for kidney which had twice the activity. Skeletal muscle had little, if any, of either of these enzymes. The ratio of the G-6-PD activity to that of 6-PGD was lower in islet (1.00) than in any other tissue studied. In liver this ratio was 1.8, in brain 2.1, ovary 2.3, gill 2.5, kidney 2.9, testis 4.2, and heart 4.4.

The relatively low G-6-PD activity observed in islet tissue is not due to the presence of an inhibitor, since the addition of islet tissue homogenate to kidney did not inhibit the activity of the latter.

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Effects of cobaltous chloride on the eggs and embryos of Ilyanassa obsoleta. JOHN B. MORRILL.

In the course of a study of the effects of the animalizing agent, cobalt, on protein differentiation in mollusc eggs, *Ilyanassa* eggs and embryos were treated with sea water solutions of cobaltous chloride (0.001–0.05 *M*) for 2–24 hours at 20–23° C. Treatment was begun at the trefoil stage and at later stages up to and including 4-day embryos with foot and shell gland anlagen. Eggs were either killed during the treatment period or developed into one of several types of abnormal larvae or into normal veligers. The main types of abnormalities included: (1) irregularly lobed, ciliated bodies filled with a mass of yolk cells; (2) veligers with a reduced body, a swollen eyeless velum filled with a mass of yolk, and rudimentary foot, shell gland and cerebral ganglia; (3) veligers with deformed but not swollen velum, eye malformations, and rudimentary cerebral ganglia, shell and mantle fold; (4) veligers with reduced velum and eye malformations. Most of the eye malformations consisted of the absence of either the left eye (100 cases), the right eye (76 cases) or both eyes (101 cases). Occasionally veligers with supernumerary eyes occurred (3 cases). The absence of an eye from one side was associated with an abnormal but not necessarily reduced cerebral ganglion. The greatest number of veligers with eye malformations developed from eggs treated at the beginning of the trefoil stage with 0.0015 and 0.002 *M* cobaltous chloride for 24 hours.

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Effect of lithium chloride on the number of eyes in Ilyanassa obsoleta veligers. JOHN B. MORRILL.

Ilyanassa eggs and embryos were treated with sea water solutions of lithium chloride (0.01–0.05 *M*) for 2–24 hours at 20–23° C. Various aspecific malformations resulted, including

shell-less veligers with reduced velar lobes and everted stomodaea. But the most striking malformations were seen in relatively normal veligers in which one of the paired eyes was absent or in which one or more supernumerary eyes were present on the right or left side or on both sides of the velum. These eye malformations were obtained frequently when eggs in the trefoil stage were treated for 24 hours with 0.02–0.03 *M* lithium chloride. Of ninety veligers with eye malformations 44% had two eyes on the right side of the velum and one on the left side; 15% had two eyes on the left side and one on the right; 16% had two eyes on one side and none on the other; and 25% had four or five eyes distributed in various combinations between the two sides. Supernumerary eyes were twice as frequent on the right side as on the left side of the veliger.

Microscopical examination of whole mounts of normal veligers revealed that the eyes with their mass of pigment granules and hyaline vesicles were closely associated with the lateral margins of the cerebral ganglia. Supernumerary eyes were similar structurally to normal eyes and were either next to the regular eyes in the region of the cerebral ganglia or else located in the region of the cerebral commissure. Regardless of their position, supernumerary eyes were always next to cerebral nerve cells. When a regular eye was absent from one side, the velar lobe and cerebral ganglion on that side were reduced.

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Uptake and transport of L-leucine by intestinal segments of Ameiurus nebulosus and Stenotomus versicolor. X. J. MUSACCHIA, S. S. FISHER AND D. WESTHOFF.

A preliminary investigation of absorption and transport of amino acids by segments of fish intestine was made. The *in vitro* everted intestinal sacs, previously described, were incubated in teleost Ringer's containing L-leucine (2.5, 5 and 10 millimolar) at 22° to 25° C. In catfish (50 animals), the middle intestinal segments showed the greatest level of uptake from the mucosal fluid and increased concentration in the serosal fluid. This held consistently for the three concentrations used, *i.e.*, 2.5, 5 and 10 millimolar L-leucine. In scup (33 animals), the uptake appeared greatest in the upper intestinal segment only with 5 and 10 millimolar L-leucine. In terms of transport the greatest increases in concentrations in the serosal compartment of both upper and lower intestine (21 animals) were obtained with the 2.5 millimolar L-leucine.

When the incubation media for catfish (12 animals) intestine were made anaerobic by gassing with nitrogen, uptake of 5 millimolar L-leucine was greatly inhibited only in the middle and lower segments. Nitrogen gassing also inhibits uptake of 5 millimolar L-leucine in both upper and lower segments of scup (9 animals) intestine.

When the intestine of catfish (6 animals) was treated with 0.1 *M* ethionine, as previously described, there was an enhanced uptake of the 5 millimolar L-leucine in all three intestinal areas. Histological studies of these segments are underway.

Each experimental series was paralleled with "blank" runs (6 to 12 animals), *i.e.*, incubation in Ringer's minus the test compound. Final values are adjusted by taking into consideration the varying amounts (non-detectable to trace amounts) of endogenously produced amino acid or sugar.

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A comparative study of D-glucose uptake and transport by intestinal segments of Stenotomus versicolor and Ameiurus nebulosus. X. J. MUSACCHIA, D. WESTHOFF AND S. S. FISHER.

Previous studies showed that D-glucose was absorbed and actively transported across intestinal wall of catfish, *A. nebulosus*, whereas in scup, *S. versicolor*, intestinal transport did not occur. Re-investigation with improved *in vitro* methods showed that D-glucose was taken up and actively transported by inverted intestinal sacs made from upper and lower sections of scup (26 animals) mid-gut. Segments from catfish (15 animals) mid-gut were also studied:

upper (adjacent the pylorus), middle, and lower (adjacent the rectum). Thus, characteristics of absorption and transport of various intestinal areas were evaluated. Evidence for active transport was taken as movement of sugar from the mucosal fluid to the serosal fluid against a concentration gradient when the initial concentrations on both sides are equal. Serosal/mucosal ratios of D-glucose were calculated: micromoles/gm. dry wt. gained in the serosal fluid divided by micromoles/gm. dry wt. lost from the mucosal fluid. In catfish preparations with starting concentrations of 5 mg.% and 10 mg.%, S/M ratios were highest for upper intestinal segments. Measures of uptake (absorption) only, showed that middle intestinal areas were most efficient.

Because catfish are subject to variation in environmental temperatures a series of 13 specimens were maintained at low temperature (10° C. at least one week) and intestinal segments were incubated with starting concentrations of 5 mg.% D-glucose at 10° C. Absorption in the upper segments was reduced about 70% and at least 95% in middle and lower segments.

In scup, using starting concentrations of 5 mg.% (found optimal for uptake and transport) and 10 mg.% D-glucose, S/M ratios were lowest in upper segments.

When incubation media were gassed with nitrogen, active transport of D-glucose was inhibited in intestinal segments of scup and catfish.

Preliminary studies of effects of carcinogenic agents were made by flushing the entire intestine of catfish with 0.1 *M* ethionine. Five days later the three intestinal areas from 15 catfish showed enhanced uptake and transport of D-glucose.

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Environmental temperature change and blood sugar change in the toadfish, Opsanus tau. PAUL FOLEY NACE AND JOSEPH E. SCHUH, S.J.

Following the observation of seasonal change in blood sugar levels of toadfish, groups of experimental animals were kept in the laboratory at temperatures maintained above and below the seasonal harbor temperatures. A large group of animals was kept at 15° C. while the sea water supply rose to 21.8° C., and smaller groups were kept within the ranges of 0–3° and 4–5° C. In winter, aquarium temperatures were raised gradually from 2° to 22° C. Blood samples were drawn from the gill arches and analyzed by modified Folin and Glucostat procedures.

In early summer, fish kept at low temperatures showed the blood sugar levels appropriate to the season of corresponding low environmental temperature. In mid and late summer, increasing numbers of fish failed to show hyperglycemia at reduced temperature. Variation in osmotic strength and photoperiod did not conclusively restore the hyperglycemic response to reduced temperature.

In May, fish kept at 5° C. attained blood sugar levels of 440 and 467 mg.% (Glucostat) without alloxan. Mean values were 171 mg.% (F) and 211 mg.% (G) at 0–3° in May, and 108 mg.% (F) and 138 mg.% (G) at 4–5° C. Controls at 10–15° averaged 42 (F) and 16 (G) in 1960, and 61 (F) and 38 (G) in 1961. June means were comparable, but July means fell to 97 mg.% for both Folin and Glucostat at 0–3° C. Through July and August, some fish maintained levels as high as 350 mg.% at low temperature, but increasing numbers showed levels equivalent to the warm water controls. In winter, fish taken from Eel Pond at –1.7° C. showed means of 152 (G) and 123 (F) after one day at 16° C. and 104 (G) and 67 (F) after two days at 21° C.

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Mechanical and electrical properties of some invertebrate visceral muscles. T. NAGAI, R. A. NYSTROM AND C. L. PROSSER.

Non-striated muscles of invertebrates in which conduction has previously been examined conduct mainly by intrinsic nerves, rarely by stretch, whereas vertebrate visceral muscles

conduct electrically from fiber to fiber. Conduction was studied in the intestines of *Venus, Spisula, Busycon, Loligo, Thyone* and *Libinia*. Average velocities in moist air were: *Venus* 1.1 cm./sec., *Spisula* 2.3 cm./sec., *Busycon* 9 cm./sec., *Thyone* 5.9 cm./sec., *Libinia* 33 cm./sec. Conduction occurred when mechanical pull was prevented in *Thyone, Venus, Busycon* and *Spisula*. Intestinal conduction was blocked by procaine (or tetracaine) in *Libinia*, not in the molluscs or holothurian. In *Spisula*, and to a less extent in *Venus*, the intestines showed both phasic and tonic contractions, both spontaneously and in response to shocks. In *Spisula* the electrical threshold for the tonic contraction was lower than for the phasic one and action potentials were associated with the phasic, not with the tonic contraction. Phasic contraction time for *Venus* intestine was 2 to 3 seconds, time for half-relaxation 15 to 40 seconds; tonic contractions persisted for many minutes. Contractions of the *Libinia* intestine were fast (0.2- to 0.4-second contraction time) and spontaneous contractions were often tetanic with an electrical spike corresponding to each rhythmic contraction. At times the electrical record consisted of a volley of fast spikes. Acetylcholine was relatively ineffective on the crab intestine but 5-hydroxy-tryptamine elicited trains of large synchronized spikes. Squid intestine and esophagus showed continuous asynchronous spike-like electrical activity. Conduction in the *Libinia* intestine appears to be by nerves and the spontaneous contractions neurogenic, while conduction in the clams, snail and holothurian appears to be from muscle fiber to fiber much as in vertebrate smooth muscle.

The glass micropipette as a mechano-transducer. M. OGATO.

If current is applied through a conventional glass micropipette, filled with 3 *M* KCl, and its tip is in contact with the surface of a rubber or polyethylene film, or a muscle (suspended in frog Ringer solution), the resistance of the electrode changes in approximate proportion to the extent of movement at the contact. This change of resistance was measured by means of a Wheatstone bridge circuit. When the polarity of the pipette is reversed, the change of resistance displayed on the CRO also reverses its sign. The exact nature of the resistance change of the pipette induced by the movement of the material in contact with its tip is under investigation.

The promptness of this resistance change provides us with a new method for the study of the relationship between membrane activity and the early phase of the mechanical response. Inserting an intracellular electrode into the frog sartorius at a point close to the mechano-transducer pipette, and recording the action potential together with the mechanical response after direct stimulation, the latency period (the time lapse between the peak of the spike potential and the onset of mechanical activity) is accurately determined. Such measurements show that the latency period of the frog sartorius at 20° C. is 1.9 msec.

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Pigmentation in Cordylophora lacustris. EDWARD E. PALINCSAR AND GEORGE SZABÓ.

Cordylophora in nature is dark brown. When cultured by Fulton's technique, the pigmentation is lost and the morphogenetic pattern of the colony changes. The nature of this brown pigment was investigated by the following tests. The pigment was insoluble in acetone, chloroform, ether, xylene, ethanol, methanol and water. It bleached within 48 hours in 10% hydrogen peroxide. There was no reversible redox color change, nor a violet color in concentrated sulphuric acid, nor a change in ammonia gas. The pigment was soluble in 0.5 N sodium hydroxide, concentrated ferric chloride and slightly soluble in formic acid and in 1 N HCl. A positive test for tyrosinase (darkening of the perisarc) was obtained with dihydroxyphenylalanine at pH 7.4 and pH 6.3. Boiled controls were negative. A weak positive reaction occurred with tyrosine incubation at pH 6.8. Homogenates of *Cordylophora* tanned gelatin with catechol and a brown pigment appeared. This homogenate also considerably speeded up the rate of melanization of DOPA (pH 7.4). Non-pigmented *Cordylophora* in Fulton's medium with the addition of tyrosine (2.5×10^{-4} *M* to 10^{-3} *M*) developed brown pigmentation *in vivo* in the perisarc after 3–5 days. No brown pigmentation appeared during

parallel treatment with phenylalanine (5×10^{-4} *M* to 10^{-3} *M*), but in thienylalanine (10^{-3} *M*) the perisarc developed a dark brown pigmentation and the morphogenetic appearance of the colony changed, approaching the pattern found in nature. The data suggest that the pigment is not a carotenoid, ommochrome or flavone. It appears that the pigment is melanin or sclerotin involved in the tanning of the perisarc. Failure of this sclerotinization results in the laboratory pattern of colonies. The possibility of natural inhibitors from the food source, *Artemia*, is being investigated. A more detailed analysis of the pigment is now in progress.

Fixing agents and the echinochrome granules of Arbacia punctulata. ARTHUR K. PARPART, EARL VAN NORMAN AND JOHN C. BERNHARDT.

Arbacia eggs were observed during the action of various fixing agents by means of the television microscope at screen magnification of 5000×. Alterations in the yolk granules, echinochrome granules, cortical granules, and nucleus were observed when the egg, fixed in view on a wedge slide, had its environment rapidly changed from sea water to a fixative solution.

Osmic acid solutions in 0.5, 1 and 2% concentrations, prepared in sea water or 0.5 *M* NaCl, all stopped protoplasmic streaming rapidly (15 to 30 seconds). Until egg opacity increased (2 to 4 minutes), the yolk, cytoplasmic, and cortical granules and the nucleus appeared well fixed. However, the echinochrome granules all exploded violently after one to three minutes.

Bouin's, Susa's, Zenker's, Zenker-formol, and Dalton's solutions, as well as 0.03, 0.3 and 3% $KMnO_4$ in sea water, caused major disruptions and explosions of cytoplasmic and cortical granules. The echinochrome granules all exploded. The egg volume increased.

Five per cent formalin in sea water and 1% $HgCl_2$ in sea water each gave good preservation of cytoplasmic and cortical granules, but after 10 to 15 minutes the echinochrome granules exploded. One per cent osmic acid in sea water added any time up to 10 minutes after this solution also caused explosion of echinochrome granules.

Uranyl nitrate (4%) dissolved in sea water stopped cytoplasmic streaming rapidly, and cytoplasmic, cortical, and echinochrome granules and nucleus were in excellent condition. Subsequent fixation by any method produced explosions of the echinochrome granules.

During the first few seconds after addition of 10% acrolein in sea water, the fixation of the egg was excellent. After 30 to 60 seconds, cytoplasmic and echinochrome granules began to explode, and at two to four minutes the cytoplasm appeared granular, with surface blebbing.

A new method of making photographic films for electron microscopic autoradiography. DELBERT E. PHILPOTT AND CLAIR E. FOLSOME.

Photosensitive films were produced by a combination of shadow-casting and vapor application. The thickness of the resulting film can be controlled by calculating the amount of silver to be used for shadowing. This amount of silver is shadow-cast directly down at 90° on a collodion filmed electron microscope grid at an estimated thickness of 50 Å. The resulting silver film is not continuous and when viewed under the electron microscope is seen to be made of very fine, individual crystalloids of silver, averaging several hundred Å. This silver is then converted to the halide, in the dark, by blowing iodine or bromine vapors over the silver surface. The resulting films are extremely thin, compared to ordinary photographic films, and since sections or other material containing radioactive tagging can be placed on the grid before this process takes place, the photosensitive film can be formed in contact with the surface of the specimen.

Electron diffraction has been used to follow the production of the silver films, their conversion to the halides, and reconversion to silver upon exposure, as well as electron microscopy of the resulting products.

Higher resolution should be possible for two reasons. The silver particles, and thus the resulting halide, are about ten times as finely dispersed as ordinary photographic emulsions and the thinness of the resulting film reduces the effect of scatter as the radioactive decay passes out through the film. Preliminary experiments with tritium-labeled bacteria indicate that this method can be used for autoradiography.

Effects of distal and proximal hydranth extracts and stem extracts from adult Tubularia on the regeneration of isolated proximal ridge regions. JOSEPH A. POWERS.

Twenty pieces from the respective adult *Tubularia* regions were homogenized in an ice bath. These homogenates were then brought to 10-ml. volumes with filtered, pasteurized sea water which contained 125 mg. of chloromycetin per liter. Centrifugation at 3400 r.p.m. for about six minutes followed. The respective supernatants were decanted into stender dishes and refrigerated for two days. The control was 10 ml. of filtered, pasteurized, chloromycetinated sea water given similar treatment. The test pieces were isolated proximal ridge regions of regenerating *Tubularia,* approximately stage 6 (Berrill and Davidson, 1948). The extracts and control were removed from the refrigerator and placed in an ice bath. Ten test pieces were then placed in each of the four solutions, *i.e.,* distal, proximal, stem and control supernatants. These stender dishes were then placed in a water bath (13° C.–16° C.). Observations were made 24–30 hours after treatment. Eighty-three per cent of the 30 pieces treated with the supernatants of distal homogenates lacked distal parts, though in most cases the gonophores and proximal tentacles were quite normal. Treatment with the proximal solutions caused 97% of 30 test pieces to lose their structure, with the coenosarcs becoming completely opaque. The latter was also true of 63% of the 30 assay pieces treated with stem homogenate supernatants. Only 17% of the control pieces failed to regenerate all of their parts normally.

Contractile responses of marine and fresh-water sponges. C. L. PROSSER AND T. NAGAI.

Oscula and "veins" of sponges contract in response to mechanical, not to electrical, stimulation. Contracting myocytes are scattered and are less than 5 μ in diameter. Contractions of *Microciona* showed a latency of 0.3 to 1.0 second, contraction time of 4 to 5 seconds, time for half-relaxation 15 to 20 seconds. Contractions of *Spongilla* were slower. Responses recorded with electrolytically tapered silver leads were of similar time-course to mechanical recordings with a transducer, whether recorded in sea water (*Microciona*) or in pond water (*Spongilla*). All contractions were local and non-propagated, but they showed summation up to a maximum response at 3 to 4 taps within one second. When calcium was omitted from artificial sea water bathing *Microciona,* the mechanical responses decreased and sometimes disappeared after 10 to 15 minutes; recovery was complete when calcium was restored. Increases in potassium in the medium to 10 times that in normal sea water for *Microciona* and to 12 m*M* in pond water for *Spongilla* did not impair the responses to mechanical stimulation. Such concentrations of potassium must have depolarized the cells. It is concluded that the contractile responses of sponge myocytes are non-propagated, that they may not involve membrane action potentials but that they involve a calcium-releasing mechanism.

The use of permanganate as a fixative for electron microscopy of invertebrate eggs. LIONEL I. REBHUN.

A variety of procedures using permanganate as a fixative for invertebrate eggs was tried. Various concentrations from .5% to 5% were used, temperatures were varied from 0° C. to room temperature, formalin or osmium in sea water were used as postfixatives, sodium or potassium permanganate was used, the permanganate was dissolved in distilled water or sea water, and finally, the pH was either uncontrolled or maintained at about pH 7.5, using either veronal-acetate or dichromate as buffers. The final mixture used was 5% potassium permanganate plus 1% potassium dichromate at about pH 7.5. Little variation was seen with different procedures as long as the concentration of permanganate was at 5%. Fixation was for ½ to 1 hour at room temperature, dehydration was with acetone or ethylene glycol and Araldite 502 or Epon 812 were used as embedding media.

The final procedure yields very uniform results, giving excellent preservation of endoplasmic reticulum, Golgi bodies, mitochondria, etc., that is, all membrane systems within the egg. The surface membrane is not as well preserved and occasionally small vesicles (blebs)

can be seen arising from *Spisula solidissima* vitelline membranes, which vesicles are not seen by osmium fixation or by freeze-substitution. Surfaces of fertilized *Arbacia* eggs are occasionally disrupted by the fixative. However, as a fixative which is consistent and easy to use, yielding excellent preservation of internal membrane systems, permanganate is superb.

Polarized control of regeneration in Tubularia by charged particles. S. MERYL ROSE.

When a distal graft of a regenerating *Tubularia* primordium is made to the distal end of a whole primordium, one is combining two differentiated, but not fully developed, distal parts and one proximal part, DDP. If this is done during the labile proximally striate stage, reorganization occurs. The first D retains its organization and continues to develop as D. The second D loses all visible signs of differentiation and becomes a P. Part of the original P after considerable loss of structure may become the base of the hydranth and the upper part of the stem. The result is DPS. The work has now been repeated but with the addition of a direct current of 25 ma and 12–30 volts across a plastic tray containing 2% agar in sea water, with a cross-sectional area of 280 mm.^{2} from distal to proximal or in the reverse direction. The current is applied for 4–5 hours and then the combinants are transferred to standing sea water at 15°–16° C. In all cases when the primordia were in the proper stage and faced the anode, (+) DDP, the result was well organized single hydranths, DPS. There were 19 cases. When the arrangement was (—) DDP, the result was always DDP. There were 18 cases. In this arrangement the distal part at the end did not control the distal part behind it and a hydranth with two sets of distal tentacles and two mouths arose. It is suggested that differentiation-controlling substances in *Tubularia* are positively charged and move from distal to proximal. When an electric current is applied in the direction that would prevent the motion of these particles along the already polarized pathway, more distal parts can no longer suppress differentiated and partially developed parts lying behind them.

DNA synthesis and cell division in elasmobranch lenses maintained in vitro. HOWARD ROTHSTEIN.

Investigations of lens epithelium employing autoradiographic procedures have indicated that it is possible to maintain both DNA synthesis and mitosis *in vitro*. If the isolated lens of the dogfish, *Mustelus canis*, is perfused at 1.25 ml./hr. with tissue culture medium (M-199, 18° C.) which has been modified by adding 2.16 gms. urea/100 ml. and 0.79 gms. sodium chloride/100 ml., the epithelial cells incorporate thymidine-H^3 after having been *in vitro* for 48 hours. In such lenses, which have been completely isolated from the surrounding tissues, mitosis ceases almost completely. Mitotic activity can, however, be maintained to a certain degree if the anterior portion of the eye is left attached to the lens during incubation. There is no apparent difference between lenses incubated in M-199 and those in elasmobranch Ringer. Parallel findings have been obtained with the skate, *Raja erinacea*. Isolated lenses incubated *in vitro* for 24 hours at 18° C. in either elasmobranch Ringer or modified M-199 manifest active incorporation of thymidine but show virtually no mitosis. There is, therefore, an apparent dissociation of cell division from DNA synthesis under these conditions. However, as in the dogfish, *in vitro* lenses which remain attached to the anterior portion of the eye continue to show mitotic activity for a long period (in this case, through 48 hours). Preliminary experiments indicate that upon incubation of the lens within the whole eye, in which a corneal window has been dissected, the lens epithelium loses its mitotic activity within 24 hours. Thus, there is an indication that the loss of mitotic activity in the isolated lens is not due to the effects of physical manipulation during the process of isolation, but rather to the withdrawal of factors which stimulate, or aid in the maintenance of normal mitotic activity. In the dogfish, cells undergoing mitosis and DNA synthesis are more numerous near the equator, whereas the skate does not show such localization.

Cytoplasmic radiation damage in Arbacia punctulata. RONALD C. RUSTAD.

Numerous examples of cytoplasmic radiation damage are known. In general, structures associated with the nucleus are more sensitive than purely cytoplasmic systems. Two studies

on anucleate *Arbacia* eggs revealed no radiation-induced mitotic delay following insemination with normal sperm. However, the anucleate cells were prepared by centrifugation, which stratifies various cytoplasmic components.

In the present study anucleate half-eggs were prepared by cutting the cells with a fine glass needle. Nucleate half-eggs divided at the same time as uncut eggs. The anucleate controls cleaved between five and ten minutes later. Irradiation of anucleate cells with x-ray doses between 17 and 60 Kr. produced significant mitotic delay. At high doses most of the embryos lysed before hatching.

These data show that the cytoplasm of *Arbacia* eggs is x-ray-sensitive, although the site of damage is unknown.

Seasonal changes in blood sugar and related phenomena in the toadfish, Opsanus tau. JOSEPH E. SCHUH, S. J. AND PAUL FOLEY NACE.

Through 18 months, some 800 toadfish were assayed for normal blood sugar (Folin and Glucostat), hyperglycemic and lethal doses of alloxan and magnitude of hyperglycemic response to alloxan. In this period, sea water temperatures varied from —1.7° C. (January) to 21.8° C. (August). Blood samples were drawn from heart and gill arches. Intracardiac injection of 10% aqueous alloxan ranged from 200 to 850 mg./kg. Simultaneous Folin and Glucostat determinations were run on each sample. The difference between the two values (expressed as mg.% glucose reducing equivalents) measured reducing substances other than glucose. Principal islet, pituitary, liver and kidney of each fish were fixed for histological study and tritiated thymidine autoradiography was used to examine variations in mitotic rates of these tissues and of skin and intestine.

As water temperatures declined in autumn, both Folin and Glucostat values rose, from August mean values of 30 mg.% (Folin) and 20 mg.% (Glucostat) to February levels of 118 mg.% (F) and 140 mg.% (G). From November through April, many fish assayed above 200 mg.% by both procedures. Only in this period have individual Glucostat values exceeded Folin values. In winter, alloxan dosages of 300 mg./kg. were hyperglycemic, while 700 mg./kg. was necessary in early summer. Doses of 850 mg./kg., which were lethal without hyperglycemia in late summer, produced no deaths in winter. Post-alloxan levels as high as 440 mg.% (G) and 417 mg.% (F), found in winter, represented increments two to three times the greatest increments common in summer.

This work was conducted with the assistance of Costante Ceccarini and Henry Laufenberg. It was supported by grant A–1129, NIAMD, NIH, USPHS, grant T–93 of the National Research Council (Ottawa), the Brown-Hazen Fund of The Research Corportion and by AEC–MBL contract.

Heat-induced local furrow rupture in Arbacia eggs. ALLAN SCOTT.

A heat treatment of one minute at 39.0 C., applied at about 40 minutes after fertilization, causes "spontaneous" breaks in the furrow cortex during cytokinesis. An analysis of the behavior of the echinochrome granules in the cortex of heated eggs shows that the sudden local out-thrust of the furrow results from the yielding of a sector of the furrow cortex. When the furrow gel parts, the granules in that area of the cortex move rapidly to the edges of the area and the endoplasm flows out to fill that sector of the furrow. The granules involved move, in one or two seconds and collect at the two boundaries of the ruptured furrow band. There is no movement of granules in directions other than around the equator. Granule patterns in the adjacent subfurrow cortex remain unaffected. The normal furrow cortex is viewed as being under a circumferential tension, perhaps due to the contraction of fibrils oriented around the equator. The rupture may involve an area only a few micra in diameter or it may involve a third of the furrow cortex. It is not a simple solation for this would not explain the directed movement of the granules. The rapid granule movement is considered to result from the parting of fibrils in one region of the furrow band and a sudden contraction of the gel on either side of the break. The repaired area is immediately integrated into the furrowing. One burst may be followed by another in the same or in another sector of the furrow, yet cells often divide successfully after several ruptures. The evidence that the

furrow is under contractile tension seems indubitable and the nearly instantaneous formation of new furrow cortex seems especially noteworthy.

Surface changes during cytokinesis in flattened eggs. ALLAN SCOTT.

It is possible to follow the shrinkage of the furrow cortex and the expansion of the non-furrow cortex during the early cleavages of the *Arbacia punctulata* egg by photographing the changing patterns of echinochrome granules. *Arbacia* eggs cleave well although flattened if they are in 70% sea water. The geometry of the flattened egg surface in relation to the mitotic apparatus is greatly altered from that obtaining in the spherical egg, since the two flat surfaces are brought close to the mitotic apparatus whose long axis is forced into the plane of the slide.

We have shown previously that the ring of furrow cortex around the equator of the spherical egg, shrinks two-dimensionally at the onset of cytokinesis. The first cortical areas to shrink during mitosis in the disc-shaped egg lie on either side of the spindle. Two triangular areas of concentrating granules are joined at their apices across the spindle equator. The area beyond the base of each triangle and lying in the presumptive furrow path, shows no sign of shrinkage at first. Cortical shrinkage proceeds outwards from the triangular areas to the edge of the disc, after which a deepening furrow cuts in from the disc margin. Granule-pattern-expansion occurs in the areas of flattened cortex covering the asters but there is no obvious expansion of the cortex that would represent the polar cortex in unflattened cells. Flattened polyspermic eggs always show an expansion of cortex in the areas covering the several asters and a shrinkage of the cortex between them. These observations are consistent with Wolpert's concept of astral relaxation and with the view that a furrow inducing substance arises from the mitotic apparatus.

Reaggregation of regions of Amaroecium zooids following their independent culture in vitro. SISTER FLORENCE MARIE SCOTT AND PRISCILLA CROWE.

This experiment was designed to test the stability of tunicate tissues in their ability to recognize, in synthetic combinations, tissues of the same histogenetic character. Young adult zooids of *Amaroecium constellatum* were divided into two regions, branchial basket and abdomen. Both regions were washed in several baths of sterile sea water (and penicillin) and then placed separately in a culture medium consisting of filtered, autoclaved sea water, dextrose, penicillin and streptomycin, with phenol red as an indicator. They were cultured for periods of twelve and twenty-four hours. They were then combined in either of two patterns: one branchial basket and one abdomen; or two branchial baskets and one abdomen, the combination of parts being minced together in the tunic of an older *Amaroecium.* The minced mass was held in place in the host test by a plug of tunic of the day-old experimental animals.

The parts that were cultured for twelve hours reaggregated and formed a single zooid in double combinations, or Siamese twins in triple combinations, in all cases where the injured surfaces of contributing parts were brought into close contact. The series of unions, whether in double or triple combination, from the twenty-four cultures reaggregated in a very low percentage of cases. Triple aggregates of such fragments minced together immediately upon dissection form Siamese twins with persisting regularity. Conclusive evidence depends upon the analysis of serial sections but it seems that, by maintaining fragments of tunicates independently *in vitro,* something is lost either from the cells themselves or from the intercellular substance that enables tissues to recognize, grow toward and fuse with tissues that share their histogenetic character.

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Osmotic regulation in the estuarine isopod Cyathura polita. EARL SEGAL AND KAY MCDONALD.

Cyathura polita were collected at low tide in the Pocasset River marsh on Cape Cod, Mass. During a daily tidal cycle the salinity of the waters flowing over the animals may vary from $\triangle$ 0.02 to $\triangle$ 0.95 (approximately 1–60% sea water based on 100% s.w. = $\triangle$ 1.70).

All animals were maintained in 25% s.w., with the natural substrate, for at least 24 hours before being placed at the experimental salinities—distilled water, natural river water at low tide (2.5% s.w.), 1%, 3.5%, 25%, 50%, 75%, 100%, 125%, and 150% s.w. Blood was picked up in 0.4-mm. diameter blood coagulating tubes through an incision made in the dorsal carapace behind the heart. Samples were taken at 3-, 24-, and 48-hour intervals. All samples were quick-frozen on dry ice and the osmotic pressure of the blood determined by a modification of the comparative freezing point technique suggested by Gross. The experiments were carried out at 22° C.

In the range of salinities to which it is normally subjected, *Cyathura polita* is a superb regulator of its blood osmotic concentration. In external salinities of 75%–1% s.w. the blood concentration falls but only within the relatively narrow limits equivalent to 75%–65% s.w. In distilled water the blood concentration is maintained at 60% s.w. for at least 48 hours and undoubtedly longer, since we have been able to keep *C. polita* in distilled water without ill effects for one week. *C. polita* does not maintain an osmotic gradient between its blood and the medium in external salinities of 75% to 150% s.w. However, in 100% s.w and above, isotonicity is not evident at the three-hour sampling although the blood of these animals is more concentrated than that of animals in 75% s.w. at the same time. By 24 hours the blood of all animals at the higher salinities is isotonic with the medium.

Animals placed in 100% s.w., 25% s.w., and river water, then weighed 1, 2, 3, 4, 8, 24, 48, and 72 hours thereafter showed no systematic weight changes. This suggests that both hypoosmotic regulation and hyperosmotic isotonicity are accomplished by the gain or loss of ions, rather than the gain or loss of water.

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Influence on the early development of three varieties of wheat of varied sea water-tap water mixtures. B. P. SONNENBLICK.

Concurrent with low dose radiation studies, the influence of water (tap, distilled, sea, and sea-tap water mixtures) on early wheat growth (Dual, Thorne and Pennoll varieties) was tested in replicate experiments. Seeds, dormant or variously pretreated, were germinated on filter paper in polystyrene dishes in which daily changes of test waters were made. Experiments continued for 6–8 days, with temperature continuously recorded, light intensity measured, dishes daily rotated according to a pattern, and pH of all fluids periodically taken. The sea water-tap water mixtures ranged from 1 : 1 to 1 : 9 dilutions. Observations were made on some 2000 seedlings, each of which was finally cut and measured from the kernel to the tip of the first leaf; root measurements were made in many instances.

The following comments may be made: (a) the mixtures form a series of diminishing alkalinity, the least alkaline favoring growth, (b) germination begins in all mixtures, from 1 : 1 to 1 : 9, but not in sea water, (c) leaf growth in 1 : 9 mixture may occasionally approximate that of controls, but controls usually average slightly longer length, (d) influence of mixtures on first leaf growth may be summarized, those from 1 : 6 to 1 : 9 sea-tap waters maintain good to very good growth, those from 1 : 1 to 1 : 3 result in non-appearance of first leaf to poor growth, while those in the 1 : 4 and 1 : 5 categories give intermediate results. This latter group of sea-tap water mixtures strongly merits further systematic study under varied conditions and with material carried as far to maturity as possible, (e) a test series made in flats with a vermiculite-peat-soil combination or vermiculite alone suggests that growth in 1 : 4 and 1 : 6 mixtures is superior to that with the same mixtures in test dishes, (f) roots of control seedlings were elongate and straight, but those developing in mixtures range from twisted, shriveled fiilaments (1 : 1 to 1 : 3) to wavy, long roots (1 : 9).

Time-lapse motion pictures of deviations in density, viscosity, and cleavage in Arbacia derived from various fertilization combinations of irradiated gametes. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Eggs and sperm were irradiated with either x-rays or 2537 Å ultraviolet rays. They were then mixed with one another or with normal gametes. Eight different fertilization combinations of irradiated gametes were thus obtained. During the first hour or two many zygotes

developing from these combinations exhibited conspicuous deviations in density and viscosity from the normal. One type of deviation was characterized by formation of irregular dense areas in both central and peripheral zones. The peripheral zone often exhibited progressive crenation. A second type was featured by sudden protoplasmic readjustments as the time for first (or later) cleavage approached. As tensions changed near the incipient furrow the relatively stiff gel zone gave way sufficiently to allow the more fluid cytoplasm to break through and bring about a different arrangement. Several adjustments of this sort might occur in quick succession. These appeared as *popping* adjustments in time-lapse motion pictures which showed *movements accelerated 32 times.* The ensuing cleavage was usually irregular, often resulting in the formation of several blastomeres instead of two. A third type was induced by strong x-ray or ultraviolet irradiation. This type was characterized by violent general internal upheaval with profound gel-sol changes accompanied by temporary peripheral bulges. The integrity of the cell was destroyed as rapid churning of cytoplasmic constituents took place with drastic alteration of intracellular organization. This was followed by massive central pigment concentration and cell death. In some instances severe irradiation of gametes effectively prevented normal junction of the egg and sperm pronuclei.

Time-lapse cinephotomicrographs of specific cases from the moment of insemination on, vividly illustrate the progress of density and viscosity changes in the three types described above. They also reveal the sequence of protoplasmic events that culminate in abnormal cleavages.

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Heavy water as a parthenogenic agent. WILLIAM SPINDEL AND PAUL R. GROSS.

The effect of D_2O upon dividing cells is delay or arrest of mitosis, depending upon the concentration of deuterium. Thus, cleavage of the fertilized sea urchin egg is blocked by > 70% D_2O-sea water, and the effect is independent of the mitotic stage. The unusual properties of D_2O as an antimitotic are (1) the catholicity of the effect with regard to mitotic stage, extending as it does even to cytokinesis, (2) its reversibility, (3) stabilization, rather than destruction, of the achromatic figure, (4) a sharp and reversible process by which the cytoplasm and the mitotic machinery become rigid, and (5) the appearance of numerous cytasters during prolonged immersion in D_2O. These properties may help to explain our recent finding that D_2O evokes parthenogenic division of eggs stored therein prior to fertilization. When unfertilized sea urchin eggs are stored in 99+% D_2O-sea water for 1–2 hours, then washed and returned to normal sea water, they cleave, in remarkably high percentages, at about 35 minutes after removal from D_2O (21° C.). These are cleavages without benefit of sperm, and they continue for many hours, most cells becoming disorganized "blastulae." The first cleavages are always multiple and irregular in such experiments, and the furrowing pattern is closely related to the distribution of the numerous cytasters which remain and grow after removal of the cells from D_2O. When the period of immersion is shorter, or the concentration of D_2O reduced, the time required for the appearance of the first parthenogenic cleavages increases rapidly; hence, for a ten-minute storage period, the first cleavage interval is 3–4 hours, and the final yield of divided cells is smaller. Parthenogenesis cannot be produced if the concentration of D_2O falls to 70% or less. The striking effects of prolonged storage in D_2O are not results of aging alone, since controls stored for the same intervals in normal sea water do not divide. The effect appears to depend, in short, upon the formation of stable cytasters, which begins in 99+% D_2O-sea water after 10 minutes of storage for unfertilized eggs. Thus, eggs stored in D_2O and then fertilized show normal cleavage in the absence of cytasters, but multiple cleavage when cytasters persist in the cytoplasm after removal of D_2O.

In vitro studies on thymidine incorporation and cell division in skate (Raja erinacea) lens. B. DOBLI SRINIVASAN AND CLIFFORD V. HARDING.

The use of the whole-mount technique and labeled DNA precursors in *in vivo* and *in vitro* studies on cell division in lens epithelium has been described. By allowing sufficient time between exposure of the epithelium to H^3-thymidine *in vivo* and fixing, it is possible to obtain

radioactive nuclear pairs in rabbit lens. These pairs represent the daughters of the originally labeled cells, all of which are visible in the whole-mount. It is thus possible to determine the time and per cent of division of all originally labeled cells. The present study represents an attempt to obtain such pair formation in an *in vitro* system, which offers the advantage of a more direct analysis.

Freshly isolated skate lenses incubated *in vitro* with elasmobranch Ringer for 24 hours at 18° C. had essentially no mitotic activity. However, when whole skate eyes were incubated, with a portion of the posterior side cut out, for 24 hours at 18° C., mitotic activity was evident. Such "half eyes" were used for the rest of this study. Mitotic figures were seen after 14, 24, 48 hours of incubation at 18° C.; after 14, 24, 48, 72 hours of incubation at 5° C. and 0° C. Freshly enucleated half eyes were exposed to H^3-thymidine in elasmobranch Ringer (Sp. Act. 3.0 C./m*M*.; conc. 5 μc./ml.) at 18° C. for two hours, rinsed with elasmobranch Ringer and incubated at 18° C., 5° C., and 0° C. Although labeled cells were seen, no radioactive pairs or radioactive mitotic figures were seen at the end of 24 hours at 18° C. and up to 72 hours at 5° C. and 0° C. In most preparations, the diameter of the nuclei undergoing DNA synthesis appeared larger than that of the unlabeled nuclei.

It is probable, at least at lower temperatures, that a long period of time is required between the onset of DNA synthesis and the initiation of cell division.

Uterine function in experimental missed abortion. H. TAKEDA AND A. I. CSAPO.

Experimental missed abortion is produced in ovariectomized and placental dislocated 24 days pregnant rabbits by prohibiting the evacuation of the uterus with a prolonged and decremental progesterone treatment. When progesterone administration ceases, the myometrium often fails to complete delivery.

Recording the electrical and mechanical activity of the pregnant uterus in the intact animal, we studied the properties of the myometrium immediately after ovariectomy and placental dislocation and during and after progesterone treatment, for a period of about two weeks. The intrauterine pressure and the electrical activity of the myometrium at three different portions were recorded by a new method. Immediately after surgery, when the uterus is strongly dominated by progesterone the electrical activity was irregular, asynchronous and its relationship to mechanical activity obscure. The active intra-uterine pressure was small and irregular. The uterus did not respond to oxytocin.

As the daily progesterone dose was decreased gradually, the electrical activity improved somewhat. However, 3–5 days after operation both the electrical activity and the intrauterine pressure diminished; they became less regular and their relationship was obscure. This decrease in uterine activity continued after progesterone withdrawal.

However, activity improved gradually during decremental progesterone treatment if estrogen was given to the animal or if the animal was treated with estrogen after progesterone withdrawal. This improvement of uterine activity facilitated the evacuation of the uterus.

These observations confirm earlier findings, namely that the excitability and contractility of the uterus is dependent on estrogen and that progesterone blocks the myometrium, resulting in asynchronous uterine activity. They also suggest an explanation for missed abortion in women and provide the basis for a rational therapy.

The nuclear apparatus of a new species of Tracheloraphis (Protozoa, Ciliata). REUBEN TORCH.

Many Trachelocercidae contain Feulgen-negative macronuclei. Raikov found that macronuclei of three species of *Trachelocerca* contain primarily RNA, although originating from DNA-containing micronuclei. A new species of *Tracheloraphis* (Dragesco, 1960) was collected from Nobska Pond (salinity 10‰) in Woods Hole. Bright-field, phase, polarizing and interference microscopy were used to study nuclei in living organisms or in temporary mounts stained with aceto-orcein (0.4% in 70% acetic acid) or with chloroform-extracted methyl green (1% in 1% acetic acid). Other organisms were fixed in Bouin's, Schaudinn's, Zenker's or Champy's and stained with dilute (1:3) Delafield's hematoxylin, the Feulgen reaction, or gallocyanin-chrome alum.

The single complex nucleus of *Tracheloraphis* sp. contains four Feulgen-negative spheres (rarely 6 or 8), each surrounded by a clear area and a membrane. Each subnucleus (sphere + clear area + membrane) measures 6–10 μ in diameter; the spheres measure 4–6 μ. Lying between the subnuclei are two Feulgen-positive micronuclei, each 2–4 μ in diameter and surrounded by a membrane. A membrane surrounding the group of subnuclei is sometimes visible in Champy's fixed specimens, but never in living organisms. Surrounding the nuclear components is an extensive area (20–40 μ) of granular cytoplasm composed of 1–3 μ Feulgen-negative spheres, which stain with hematoxylin and gallocyanin-chrome alum. The sharp delineation of the granules from the remaining cytoplasm suggests that they belong to the nuclear complex. In most organisms, the subnuclear spheres produce weakly birefringent, plate-like crystals, generally rectangular but sometimes dumbbell-shaped or irregular. The crystals occur in matched pairs; if an arrow-shaped crystal is found in one subnucleus, an identical crystal is present in another subnucleus. During nuclear division, the micronuclei divide, forming four. Two of these, together with two of the subnuclei, migrate posteriorly. Prior to cytokinesis, the micronuclei divide again, two becoming functional micronuclei and two forming subnuclei. These observations and preliminary studies with DNase and RNase indicate this to be a direct transformation of DNA into RNA.

Differentiation of species of Diophrys by means of silver staining. REUBEN TORCH AND LINDA HUFNAGEL.

During a systematic survey of the hypotrichous ciliates of Woods Hole, five species of *Diophrys* were collected, three marine (salinity of 31‰) and two from brackish water (salinity 10‰). Routinely, the organisms were fixed in Schaudinn's and stained with dilute (1:3) Delafield's hematoxylin or by means of the Feulgen reaction. Except for differences in size and minor morphological characteristics, the animals were essentially similar: all had 7 right ventral, 5 anal, 2 left ventral and 3 caudal cirri. One species contained numerous macronuclei, while all the others had two with their associated micronuclei. While morphological characters suggested that the organisms represented separate species, species differentiation based on external morphology is difficult. Therefore, we resorted to the Chatton-Lwoff silver technique for the infraciliature which has proved important in taxonomic studies on other ciliates. The technique employed was essentially identical to that used by other workers, except that rinsing the organisms in dilute sea water (1:2) before embedding in saline gelatin gave enhanced results. (The saline rinse also improved the staining of other ciliates which are normally difficult to impregnate with silver.) Silver-impregnated slides were exposed either to sunlight or to ultraviolet light (GE H-4 lamp). Exposure time was determined by microscopic examination of the specimens and was generally around 30 minutes.

In *Diophrys*, silver staining reveals a dorsal infraciliature which is constant within each species. Of the five species studied, one has 9 kineties, one has 5, two have 4, and in the fifth species, each kinety is reduced to two or three widely separated groups of two or three kinetosomes in a row. Fibrillar connections between kineties were not observed in any of the species.

In addition to providing a means for identifying species of *Diophrys* (when used in conjunction with other morphological criteria), variations in number of kineties and kinetosomes gives insight into evolutionary relationships within the group.

A method for determination of oxygen uptake and respiratory quotients in carbon dioxide-air mixtures. DAVID M. TRAVIS.

This method employs simple apparatus for simultaneous determination of oxygen uptake and carbon dioxide output in the presence of wide ranges of carbon dioxide in air. The principle involves the sampling of gas from a reaction chamber of known volume. A 30-ml. syringe fitted with a three-way stopcock or a rubber stopper provides a vessel of variable volume which is convenient for gas sampling by pipette or syringe and needle. It will contain a variety of marine invertebrates. *Golfingia gouldii*, *Thyone briareus* and *Mytilus edulis* have been used. The simplest procedure with only a gas phase is described, although both liquid and gas phases can be employed. The syringe is filled with the animal and immersed in a water bath at constant temperature. Gas is sampled initially and at suitable intervals. Analysis can be done for

carbon dioxide and oxygen using the ½-cc. method of Scholander. The changes in concentration of these gases and the initial and final gas volumes are used to calculate oxygen uptake and carbon dioxide output. Initial volume is set at a calibrated mark with gas brought to the temperature of the bath. Changes in gas volume occur during an experiment which may seriously affect results. This difficulty is met by measuring changes in concentration of nitrogen or "inert" gas in the system. In this way the final volume is estimated indirectly. A formula can be used to correct the final oxygen and carbon dioxide concentrations. Equilibration of the animal with the gas may require repeated gassing over several hours. Great care must be exercised in the sampling and analysis of gases to avoid contamination or loss. The method is relatively simple in theory and is adaptable to a variety of biological materials and analytical techniques.

Factors affecting germinal vesicle breakdown in Pectinaria (Cistenides) gouldi. KENYON S. TWEEDELL.

Prior to shedding, the eggs of *Pectinaria* are tightly packed but freely suspended in the coelomic fluid. When shed into sea water, mature eggs continue maturation with breakdown of the germinal vesicle (G.V.), nucleolus, and formation of the first maturation metaphase. G.V. breakdown begins from six to ten minutes post-shedding, the time varying with the season, individual animals, temperature, light and other factors. The process lasts 2.5 to 3 minutes and is completed in all eggs by 15 to 20 minutes post-shedding. Since breakdown may be caused by a release from the internal environment or reaction with the external environment, several possibilities were investigated.

Animals were carefully dissected from their sand tests in Moore's calcium-free sea water, washed repeatedly and induced to shed into Ca-free, artificial (MBL formula) and natural sea water. Nuclear breakdown occurred in all solutions, although it was retarded in both artificial solutions.

To test for the possible release of calcium from the egg as the activating agent, eggs were shed into an isotonic (0.30 *M*) sodium citrate solution as well as hypo- and hypertonic solutions. Germinal vesicle breakdown occurred concurrent with controls (+ 9 minutes) and the eggs remained stable for 30 minutes in the isotonic solution.

Eggs were next shed into hypertonic or hypotonic artificial sea water (plus or minus 100 millimoles NaCl), artificial sea water and natural sea water. Nuclear breakdown again took place in all solutions but was delayed in the artificial solutions.

Animals were again dissected from their tests, washed, and blotted dry with bibulous paper. The coelomic fluid containing eggs was then either collected from the posterior gonopores or removed with a hypodermic needle. The remaining eggs were shed into natural sea water. Normal nuclear breakdown of eggs in the extracted coelomic fluid took place repeatedly as in the controls. Thus, removal of eggs from the coelom rather than external environmental factors appears to trigger nuclear breakdown. Possible causes to be tested are CO_2/O_2 ratios and hydrostatic pressure.

Studies on the site of action of alloxan: effect of alloxan on the permeability of the toadfish islet cell membrane to mannitol. DUDLEY WATKINS, S. J. COOPERSTEIN AND ARNOLD LAZAROW.

Previous studies have suggested that alloxan does not enter the cell but that it may exert its diabetogenic effect by acting on the beta cell membrane. The present investigation was undertaken to determine the effect of alloxan on the permeability of the cell membrane *in vitro*. Since C^{14}-mannitol does not enter most cells, it was used as an indicator of the integrity of the cell membrane.

Toadfish islets were removed, decapsulated, cut in half, and incubated at 0° C. in an isotonic medium containing C^{14}-mannitol, with and without added alloxan ($2.5 \times 10^{-4} M$). (The diabetogenic dose of alloxan in the rat, 40 mg./kg., is equivalent to 2.5×10^{-4} moles/kg.) The islet slices were removed after various incubation periods (0, 5, 10, 15, 30, 60, 120, and 180 minutes), and rinsed momentarily in a medium containing non-radioactive mannitol in

order to minimize surface contamination. The slices were weighed and homogenized in water, and the C^{14} content per mg. of tissue was determined.

The results showed that at maximum equilibration the C^{14}-mannitol content of the tissue was 30% of that in the medium. With the amount of tissue used, equilibration was almost complete at 10 minutes, whereas at 5 minutes the C^{14} content was 22% of that in the medium. Alloxan markedly increased the penetration of C^{14}-mannitol; at 5 minutes the C^{14}-mannitol content of the tissue was 40% of that in the medium, at 10 minutes it was 49%, and at maximum equilibration it was 53%. The same concentration of alloxanic acid (a non-diabetogenic decomposition product of alloxan) did not influence the penetration of C^{14}-mannitol. In contrast to islet, the permeability of heart, kidney, brain, gill, muscle, and liver was not affected by this concentration of alloxan. These data are therefore consistent with the hypothesis that alloxan produces its diabetogenic effect by damaging the beta cell membrane.

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Organismic responses to differences in weak horizontal electrostatic fields. H. MARGUERITE WEBB, FRANK A. BROWN, JR. AND THOMAS E. SCHROEDER.

Snails, as they emerged from a south-directed exit to an asymmetrical field, black to left and white to right, were subjected to changes in electrostatic field. The field was modified by large vertical copper plates four inches to right and left, either shunted together by direct wire connection (control) or subjected to a 45-volt difference, +L −R or −L +R, yielding about 2 volt/cm. addition or subtraction from whatever ambient terrestrial E-W vector obtained at the time. Experimental series were run at various times of day. About half the series comprised C (control), +L, +R, C, +L, +R, C, +L, +R, C and in the remainder the experimental fields were in the reversed order (C, +R, +L, . . .). The average differences between paths in the experimental fields and the average of the two adjacent controls were determined. The +L field produced, in 52 such series, 18% greater deviation ($P < .02$) from controls than +R. The effect of the +L field was stronger for those series +L, +R (+31%) than for the alternative series, +R, +L (+4%), suggesting a persistent effect of the fields on the snails.

In a shorter set of comparable experiments conducted concurrently, in which the snails were directed east, the +R field possessed a stronger influence, 13% more than the +L field, and comparable, when the +R field directly followed a control it was more effective. In the C +R, +L series the +R was 20% more effective than +L, and in the C +L, +R series 5% more so. The data of east-directed snails, though still too few to establish good statistical probability, do, however, clearly suggest an interrelationship between magnetic and electrostatic orientational responses.

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Allometric growth in fish. CHARLES G. WILBER.

In an attempt to ascertain more precisely the mechanism of action of selected psychotropic drugs, studies of the effects of some of them on normal growth and development in fish were initiated. Previous work showed that lysergic acid diethylamide in various concentrations modified the development of the heart in *Fundulus*. This fact suggested a more detailed study of a number of species. In the toadfish *Opsanus tau*, ranging in weight from 30–600 grams, the heart weight varied linearly with body weight. The coefficient of correlation was 0.9. If all the data were combined to get mean body weights and mean heart weights, it was calculated that in *Opsanus* the heart weight accounts for about 0.1% of the body weight. Comparisons were made with other marine and fresh-water fish. In the glut herring, for example, heart weight accounts for 0.18% of the total body weight. The average value for heart weight, as per cent of body weight, based on the 12 species available in this study, was 0.11. A similar average for mammals is about 0.84%. Comparisons of the same sort are being calculated for brain, eyes, gut and liver. With this basic information available, it should then be possible

to evaluate the biological effects of various chemical agents in terms of the modifications induced in organ weight: body weight ratios in fish.

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The effect of actinomycin D on the development of Arbacia eggs. ALEXANDER WOLSKY AND MARIA DE ISSEKUTZ WOLSKY.

The substance, known to be carcinostatic in some cases (kindly supplied by Merck, Sharp & Dohme), was used in 2.5 mg. per 100 ml. concentration. Treatment of sperm suspensions with this solution before fertilization for 3 to 4 hours and subsequent transfer to sea water produces no ill effect on fertilization and development. A similar pre-treatment of unfertilized eggs, although it apparently does not interfere with the subsequent fertilization in sea water (membrane elevation occurs promptly), delays the formation of the first cleavage furrow by 10 to 18 minutes in comparison to controls. (Normal time interval between fertilization and first cleavage under the conditions of observation: 40 minutes.) This marked delay has no influence on the formation of the furrow, which is in most cases normal. (Abnormalities were discarded, as they occur occasionally also in controls.) Later development of the pre-treated eggs is also sluggish and they never recover completely. Tetrahedric plutei are formed but the growth of the arms is stunted. Fertilization of eggs in actinomycin without pre-treatment has no immediate effect (except a much slighter delay of the first cleavage) but keeping them afterwards continuously in the solution leads to retardation and abnormalities of the cleavages and death before the gastrula stage. On the basis of these observations and earlier (unpublished) studies on the effect of actinomycin on onion root tip cells, it is suggested that the substance produces abnormal conditions in the nuclei, which manifest themselves both in chromosome aberrations (elongations and breakages, observed in onion root tips) and delayed cleavage divisions (delay of the fusion and amphimixis of pronuclei?). However, an effect on cytoplasmic constituents (kinetic apparatus) must be also assumed as the contrast between pre-treatment of sperm and eggs indicates. A microscopic study of fixed eggs to clarify these questions is in progress.

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Effect of certain adrenocorticoids on blood sugar and liver glycogen of skate. PAUL A. WRIGHT.

Apparently the only attempt to relate adrenocorticoids to carbohydrate metabolism in elasmobranchs was by Hartmann and co-workers in 1944. They showed that liver glycogen fell somewhat following interrenalectomy in skates (although the enormous range of values for intact animals made this difficult to prove statistically), but could not demonstrate any consistent lowering of blood glucose in interrenalectomized animals.

Our studies during the past two summers have indicated that certain adrenocorticoids, active in carbohydrate metabolism in mammals, at least, are of only minimal importance in carbohydrate metabolism in *Raja ocellata*. In 2 of 6 animals stunned by a blow on the head, intra-arterial injection of cortisone (2 mg./kg.) raised the blood sugar by only 32 mg.% in 30 minutes, and this value dropped to +17 mg.% by 60 minutes. Corticosterone, prednisolone, and hydrocortisone, in the same dosages, did not raise blood glucose levels significantly.

Daily intramuscular injection (5 or 10 mg.) of hydrocortisone in normal fasting animals raised the blood glucose level by only 10 mg.% in 24 hours, but cortisone in the same dosages was without effect. Liver glycogen, determined by a modified anthrone procedure 48 hours after the first of two daily 5- or 10-mg. intramuscular doses of hydrocortisone or cortisone, was not altered from that of saline-injected controls.

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Optic nerve responses of squid, Loligo pealii. ROBERT WURTZ.

Recordings from squid optic nerve by MacNichol and Love were verified and extended, and a more stable method of restraining the intact squid was developed. In the present method

only the head was restrained, leaving the mantle entirely free to perform respiratory movements. Under .3% urethane the squid was placed, back down, on cork board, and four pins were inserted into the head: one on each side just anterior and medial to the eye, one on each side lateral to the optic lobe in the lateral extension of cartilage. Posterior pins were not so tight as to pressure-block the underlying respiratory nerves. The squid was then submerged in freely running sea water at 20° C. and remained vigorous, often for 24 to 36 hours. With steel microelectrodes inserted through a separately held syringe needle, optic nerve responses were held even during siphon squirts. During light presentation initial slow oscillations and a sustained background discharge of impulses occurred. Bright light (500 mμ) produced clear oscillations of approximately 40-msec. latency and 1.4-mV. amplitude while light in a more physiological range produced very slight oscillations with latency above 55 msec. and .4-mV. amplitude. Number of oscillations varied both with light intensity and position of electrode. With D.C. coupling, oscillations were seen to occur on the rising phase of the ERG. Section of the optic nerve between the electrode and the optic lobes did not eliminate the oscillations. The oscillations later disappeared, usually within 30 minutes, as did the ipsilateral but not contralateral ERG. A knife cut between the electrode and the eye abolished immediately all nerve responses to light. Prussian blue marks left by microelectrode lesions were found to lie in the optic nerve. It is concluded that background discharge and oscillations arise in afferent optic nerve fibers. The oscillations possibly arise from partial synchronous discharge of large numbers of afferent fibers.

The nucleic acids of transparent tissues. S. Zigman, B. Carlsson, J. Kielich, E. J. Allaway and William Stone, Jr.

In this study, the distribution of RNA and DNA; the uptake of P^{32} (phosphate) into the nucleotides of RNA; and the nucleolytic enzyme activities of several transparent tissues were examined. Since nucleic acids are important in protein synthesis, it is possible that their nature and metabolism influence the formation of collagen, producing transparent—rather than opaque—tissues.

Sections of embryonic and adult dogfish corneas and shark corneas, stained by Feulgen technique (for DNA) and methyl green-pyronin (for RNA) showed that most of the RNA and DNA was localized in the epithelium of the cornea, very little appearing in the stroma.

Paper electrophoresis was employed to separate the nucleotides of RNA extracted from corneal epithelium and squid skin by aqueous phenol. A nucleotide pattern similar to that of phenol-extracted yeast RNA was found, whereas no such pattern appeared for nucleotides of corneal stroma.

P^{32} (phosphate) was found to be incorporated into all four nucleotides of corneal epithelium and squid skin RNA. These tissues were incubated at 32° C. in Ringer-bicarbonate medium (pH 7.4) containing 5 μc. of P^{32} (phosphate) for two hours. However, positively identified nucleotides containing radioactivity were not found in stromal tissue.

Ribonuclease and deoxyribonuclease activities of stromas and epithelia were measured by the release of ultraviolet absorbing materials at 260 mμ from yeast RNA. The results of these experiments are as yet incomplete.

This work shows that the epithelium of the normal cornea contains nearly all of the nucleic acids of the whole tissue. RNA metabolism, as measured by P^{32} (phosphate) incorporation into nucleotides, occurs only in the epithelium. Further work to compare the nucleic acid metabolism of these normal transparent tissues with opaque, chemically treated, and irradiated tissues is planned for the future.

Structural and functional recovery of the mitotic apparatus following disorganization by high pressure. Arthur M. Zimmerman and Douglas Marsland.

The mitotic apparatus (spindle-aster-chromosome complexes) were isolated from *Arbacia* eggs subsequent to treatment with high pressures (2000–10,000 lbs./in.^{2}) applied at metaphase of the first cleavage division. Subsequent to the compression period (1–10 min.) the eggs were fixed in cold 35% ethanol, either immediately, or after a 1–10-minute recovery period. Later, isolations were effected by selective solubilization in 1% digitonin.

As reported previously, pressure treatments of appropriate intensity and duration effect a drastic disorganization of the linear and radial structure of the spindle-aster complex and a severe dislocation of the chromosomes. One minute of compression in the range of 8000 to 10,000 lbs./in.^{2} suffices to abolish all traces of fibrous structure and to cause a clumping of the chromosomes near the center of the spindle remnant. With longer treatments, the spindle area becomes shorter and the astral remnants are eroded. At the 4000 lbs./in.^{2} level, fairly normal structure endures for some 2–3 minutes, whereas at 2000 lbs./in.^{2} an approximately normal structure persists for 10 minutes (perhaps longer), although the chromosomal movement is definitely retarded.

Post-compression recovery, as judged by a study of isolations fixed at various times after treatment, is longer with more drastic degrees of disorganization. An apparently normal fibrous structure in the spindle and astral regions and a normal metaphase arrangement of the chromosomes reappears within 5 minutes after a one-minute compression at 6000 lbs./in.^{2}, and there is a corresponding (6 minutes) delay in onset of furrowing in the treated eggs. At a pressure of 10,000 lb./in.^{2}, maintained for one minute, only partial recovery is observed at 5 minutes, but full recovery appears to occur at 10 minutes. Moreover, such eggs begin furrowing just 10 minutes later than untreated controls. This indicates that structural recovery must occur before function can be resumed.

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LALOR FELLOWSHIP REPORTS

Fine structure of an unusual spermatozoan in the flatworm Plagiostomum. A. KENT CHRISTENSEN.

Spermatozoa from the alloeocoel flatworm *Plagiostomum morgani* Graff were studied by light and electron microscopy. The sperm are spindle-shaped, tapering at each end into slender processes which are respectively about 40 μ and 10 μ long. The fusiform nucleus contains a dense core of unknown nature which protrudes from the nucleus at that end of the sperm which bears the shorter slender process. The entire surface of the sperm is covered by a pellicle consisting of the plasma membrane closely underlain by parallel fibrils or tubules, about 200 Å in diameter and spaced 700 Å apart, which pursue a spiral course up the body of the sperm. This pellicle closely resembles that described by others in trypanosome flagellates, where it may be responsible for characteristic body contractions. The cytoplasm around the middle portion of the nucleus contains whorls of flattened cisternae, resembling endoplasmic reticulum (without ribosomes), which extend out to insert along every other or every few pellicular fibrils. The rest of the cytoplasm contains mitochondria, but otherwise is homogeneous in appearance. No evidence was seen of a flagellum or of undulatory membranes.

Sperm received in copulation are transported into the general connective tissue, in which all body structures, including the ova, are embedded. The sperm wander in this tissue throughout the body, and many congregate around the eggs to effect fertilization. Since the connective tissue is a continuous mass of fixed parenchymal cells, without intercellular spaces (Pedersen, confirmed here), the sperm are wedged in between cells during these travels, and must presumably proceed by some sort of wriggling motion. Flagella or undulatory membranes would seemingly be useless under these circumstances. It is suggested that the specialized pellicle may be the basis of motility, perhaps through differential contractions of the pellicular fibrils over the surface of the sperm.

Further investigation of egg jelly dispersal by Arbacia sperm extract. R. R. HATHAWAY AND L. WARREN.

We have described previously how *Arbacia* sperm can be extracted with sodium lauryl sulphate to yield a solution which causes egg jelly-coats to disappear in 15 to 60 minutes. The evidence suggested that the jelly-coats were being dispersed rather than being precipitated

to the cell surface. We have now employed S^{35}-labelled *Arbacia* eggs to obtain additional evidence for the solubilization of the egg jelly-coat.

Arbacia eggs labelled with S^{35} were mixed with the active sperm extract. The subsequent disappearance of the jelly-coats of the eggs was accompanied by the appearance of radioactive materials in solution. Two other mixtures served as bases for comparison: (1) labeled eggs treated with acidified sea water in order to dissolve the jelly-coats completely, (2) labeled eggs treated with sea water. In four experiments, supernatant solutions of eggs treated with sperm extract contained radioactivity equal to 74%, 126%, 101%, and 111% of the radioactivity released by the eggs in acidified sea water. Since the jelly-coat contains a large proportion of sulphate groups, the appearance of S^{35}-labeled materials in solution would seem to confirm that these sperm extracts cause the solubilization of the gelatinous material. Two of the above values (126%, 111%) suggest that sulphur-containing materials from within the egg may be released following exposure to sperm extract. S^{35} in dissolved materials did not diffuse through cellophane.

After one hour there were small amounts of radioactivity in the control supernatant solutions of eggs mixed with sea water. When labeled eggs and sperm extract were kept for one hour at 0° or 11° C., the jelly-coats of the eggs did not disappear, nor did the supernatant solutions contain more radioactivity than did control solutions. Sperm extracts were inactivated when heated for two minutes at 80° C. The jelly-dispersing agent in sperm extracts can be recovered after precipitation by 30% ethanol at 0° C.

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